Evaluation of black soldier fly (*Hermetia illucens*) larvae as an alternative protein source in pig creep diets in relation to production, blood and manure microbiology parameters.

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

In the animal nutrition industry it has become a necessity to seek sustainable and alternative protein sources for animal production. As an alternative protein source, insect meals have been reported to have various beneficial effects in both production and health of animals. Thus, the aim of this study was to investigate the potential of black soldier fly (*Hermetia illucens*) larvae, grown on kitchen waste, as an alternative protein source in pig creep diets. The current trial included two treatment diets, i.e. a control diet containing no black soldier fly larvae meal (BSFLM) and an inclusion diet containing 3.5% BSFLM of the total diet. These diets were fed to 315 pure bred Large White and Landrace piglets from 10 to 28 days of age in a four week phase-over feeding scheme.

The BSFLM contained, on a dry matter basis, a crude protein content of 35.9%, 48.1% crude fat, 6.5% crude fibre and 7.8% ash. Both the treatment diets were formulated to contain similar chemical compositions with a crude protein content of 22%, 6% crude fat, 2-4% crude fibre and 4-6% ash, as this provides for the piglet's requirements (PIC, 2008). The first part of this study was to investigate the effect of larvae meal inclusion on the production parameters of the piglets. There were no significant ($P>0.05$) differences achieved for cumulative feed intake, 0.276 kg and 0.282 kg, and average daily gain (ADG), 0.203 kg and 0.199 kg, for the control and inclusion diets per piglet, resepectively. It was concluded that the BSFLM sustained normal growth and development of the young pigs and could be effectively utilized to partially replace other protein sources.

In the second phase of this study the effect of the BSFLM on the piglet blood parameters, specifically on immunology and mineral bioavailability characteristics, was investigated. There were no significant ($P>0.05$) differences observed in the haematological and biochemical concentrations (refer to Chapter 4 results), however, the inclusion diet showed increasing levels for both Haemoglobin (HGB) and Haematocrit (HCT) over the trial. Although not statistically different, this phenomena may have biological value as higher values may be correlated with better oxygen binding capacity and transport of the oxygen to the tissues of the body. These results may also be considered as an indication of immunological stress, however, the animals showed no physical signs of distress when compared to the control diet. Due to the issues of both a dilution effect and sample collection mediated stress experienced during the data collection, the exact correlation between the inclusion of BSFLM and obtained HGB and HCT values (immunological influence) could not be pin pointed. Therefore, further research is needed to validate the results achieved in this part of the study.

The third and final part of this study investigated the effect of BSFLM on the piglet manure microbiology (bacterial shedding load) and texture. However, due to the unintended administration of antibiotics which had a significant ($P<0.05$) influence on both treatments' second collection, the results could not provide for valid conclusions. Therefore, further research is required to discover any possible effects associated with the inclusion of BSFLM on piglet manure matter.

Although there were no beneficial effects on the blood and manure parameters, the fact that similar results were achieved between the control and inclusion diets leads to the conclusion that BSFLM could be regarded as a safe protein source that can be utilized to partially replace other traditional sources in the ability to sustain piglet performance, with no adverse effects. However, due to the fact that antibiotics were administered, the negative effects may have been neutralised.

Opsomming

Die soeke na alternatiewe en volhoubare proteïenbronne vir diereproduksie raak ‘n al groter dryfveer in diereproduksie. As ’n alternatiewe proteïenbron het insekprotein baie aandag gekry en is bewese in beide die produksie en gesondheid van diere. Die studie is gevolglik gedoen ten einde die waarde van die venstervlieg (Hermetia illucens), gegroei om kombuisafval, as alternatiewe proteïenbron in varkkruipvoer te evalueer. The huidige studie het twee behandelings ingesluit naamlik ’n kontrole dieet met geen venstervlieg meel in nie en ’n behandelingsdieet met 3.5% venstervliegmeel in. Hierdie voere is gevoer aan 315 Grootwit en Landras sogende varkies vanaf 10 tot 28 dae ouderdom in ’n vier weke oorgangs stelsel.

Proksimale analises is uitgevoer op die larwemeel en op droë materiaal basis bevat die larwe meel ’n ruproteïen inhoud van 35.92%, 48.09% ruvet, 6.53% ruvesel en 7.79% as. Die insluiting van larwe meel in die voere van sogende varkies en die gevolglike uitwerking op produksieparameters is vervolgens getoets. Daar was geen betekenisvolle verskille in gemiddelde daaglike inname of gemiddelde daaglike toename nie. Dus kan daar aanvaar word dat larwemeel suksesvol in voere van sogende varkies ingesluit kan word sonder enige nadelige effek.

Die tweede gedeelte van die studie het gekyk na die invloed van die teenwoordigheid van die larwemeel op die bloedparameters, spesifiek immunologie en minerale biobeskikbaarheidseienskappe. Geen betekenisvolle verskille is waargeneem vir die haematologiese of biochemiese konsentrasies van die bloed nie behalwe vir Haemoglobien en Haematokritvlakke wat hoër was vir die groep wat larwemeel ontvang het. Hierdie verhoogte vlakke is gekorreleer met verbeterde suurstofbindingsvermoë en suurstofdravermoë. Verdere navorsing op die gebied word aanbeveel aangesien bloedkolleksie stres en verdunnings effekte vermoedelik tot swakker resultate gelei het.

Die derde deel van die studie het die effek van larwemeel op fekale mikrobiologie bepaal. Die tegnieke is uitgevoer maar as gevolg van onbeplande toediening van antibiotika is die resultate nie konkreet nie en word daar aanbeveel dat verdere navorsing op die gebied gedoen word.

Uit die resultate word daar dus aanvaar dat die gebruik van larwemeel in kruipvoere ’n veilige en aanvaarbare alternatiewe proteïenbron is wat in die plek van onvolhoubare proteïenbronne gebruik kan word en dat dit nie enige nadelige effek op klein varkie produksie sal hê nie.

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Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

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Abbreviations

AA	Amino Acid
ADG	Average daily gain
AMP	Adenosine Monophosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists International
ASAE	American Society of Association Executives
BASO	Basophil
BSE	Bovine Spongiform Encephalopathy
BSF	Black soldier fly
BSFLM	Black soldier fly larvae meal
CBC	Complete Blood Count
CBV	Circulating Blood Volume
CF	Crude fibre
CFU	Colony Forming Units
CP	Crude protein
DAFF	Department of Agriculture, Forestry and Fisheries
DGS	Distiller's Grain with Soluble
DM	Dry matter
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EOS	Eosinophil
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FI	Feed Intake
fL	Femtolitre
GLM	General linear models
HCT	Haematocrit
HGB	Heamaglobin
Ig	Immunoglobulin
ISO	International Organization for Standardization
LYM	Lymphocyte
LSM	Least Square Mean
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
ME	Metabolisable Energy
MONO	Monocyte
MPV	Mean Platelet Volume
NEU	Neutrophil
NFE	Nitrogen-Free Extract
NRC	National Research Council
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
pg	Picogram

PIC	Pig Improvement Company
PLT	Platelet
RBC	Red Blood Cell
RDW	Red blood cell Distribution Width
spp.	Species
Staph.	*Staphylococcus*
TNTC	Too Numerous Too Count
TVC	Total Viable Count
USP	United States Pharmacopeia
WBC	White Blood Cell
WIC	White blood cell impedance count
WOC	White blood cell optical count

Chapter 1

General introduction

The world population is increasing rapidly and is predicted to reach 9 billion by the year 2050 (DESA, 2009). This is creating an ever-increasing demand for food and thus placing substantial pressure on the food industry to provide for the human population (Cribb, 2010; Dar and Gowda, 2013). Along with this population increase there is also the associated increase in global disposable income, which leads to a subsequent increase in the demand for animal protein (FAO, 2009). This increase reflects an expected continual rise in nutritional costs experienced by the animal production industry (FAO, 2009) due to the underlying controversy developing for the distribution of the limited plant protein sources between animal and human consumption (FAO, 2009). However, since livestock is one of the primary protein sources for human consumption that provides as an essential source of vital amino acids, emphasis is also placed on the demand for livestock meat (Hoffman and Cawthorn, 2012). This in turn creates a vicious circle in which intervention is required through global adaptation. These concerns are amplified further by the global climate change, which may lead to the reduction in crop yields over the next 50 years (Dar and Gowda, 2013). The challenge of feeding the world's population may only be addressed by an increase in global agricultural production of 70 to 100% by the year 2050 (Bruinsma, 2009). This increase in production requires the improvement in the efficiency and cost-effectiveness of food production systems with minimal effect on the environment (Berg *et al.,* 2013). Thus, agricultural investments are required to improve the utilization of limited resources, such as land and water, and extensive research should be conducted into sustainable production systems to counteract the adverse effects of climate change so as to provide and sustain global food security (Dar and Gowda, 2013). Insect meals may serve as one of the options to achieve effective sustainable production (Newton *et al*., 2005; Ijaiya and Eko, 2009; Hassan *et al*., 2009).

To feed the growing population, the global agricultural industry would have to increase production to achieve an output of approximately 200 million tonnes of livestock meat per annum (Bruinsma, 2009). Thus, increasing demand will lead to continually increasing prices for these meat proteins (Hoffman and Cawthorn, 2012). Pork is considered one of the most popular livestock meats; approximately 44% of global meat protein consumed is derived from pork and pork products (FAO, 2001). Pig production represents one of the most economical means of bridging the supply-demand gap of animal protein due to their high fecundity and high feed conversion efficiency (Machebe *et al*., 2009). They are also early maturing, with a short generation interval and have a relatively small space requirement allowing for intensive production (Machebe *et al*., 2009). However, some of the main plant protein sources utilized in pig nutrition are cereal grains, which include maize and soybean meal (Reese *et al*., 1995). Cereal grains are also utilized for human consumption and/or for the production of biofuel (Biswas *et al*., 2011), thus, these three industries are competitors for these crop commodities. Therefore, there is an incentive to find alternative protein sources for pig production that are not in competition with the human food supply or biofuel industry.

Insects are consumed naturally by free range livestock and wild animals on a daily basis. Insects have high feed conversion efficiencies and act as bio-transformers, converting organic waste to produce larvae, prepupae and pupae of high nutritional value (Diener *et al*., 2009). Insect meals are high in protein (30-80%), fat (14-50%) and in some minerals, and the variations in composition are correlated to differences in the species (Newton *et al*., 2005; Pieterse, 2014), age at harvest (Calvert

and Martin, 1969; Inaoka *et al*., 1999; Aniebo *et al*., 2008), method of drying (Fasakin *et al*., 2003) and larval feed substrate (Newton *et al*., 1977; Pieterse, 2014). Thus, insect meals have been studied as potential feed ingredients in commercial animal diets and have resulted in good growth performances, without compromising carcass characteristics (Ijaiya and Eko, 2009; Hassan *et al*., 2009; Barroso *et al*., 2014; Pieterse *et al*., 2014). Insect larvae inclusion has also been reported to decrease the incidence of metabolic skeletal disorders in broilers and improve the overall health of the birds (Pieterse, 2014; Pieterse *et al*, 2014; Uushona, 2015). This phenomenon possibly correlates with the adequate amino acid profile and a high calcium and phosphorus content, which are essential for proper bird growth and skeletal development (Pieterse *et al*, 2014; Uushona, 2015). Insect larvae have been associated with the reduction of organic waste biomass by up to 60% and the reduction of waste moisture, thus, providing a solution to the problem of waste disposal (Newton *et al*., 2005; Diener *et al*., 2009; Kim *et al*., 2011). The organic waste, after the high protein recovery by treatment with larvae, is also no longer 'too-rich' to apply to crops or to be utilized as compost (El Boushy, 1991; Li *et al*., 2011). Furthermore, larvae have been reported to reduce certain pathogens within the manure by modifying the microflora of the manure matter (Bondari and Sheppard, 1987). This limits the possible transmission of these pathogens to a host through contact (Bondari and Sheppard, 1987). Therefore, insect meal could benefit the animal nutrition industry and form a component of other production systems to increase its economies of scale; promoting an incentive to undertake insect meal production and utilization.

Most research on insect meals as an alternative protein source in animal nutrition has been on the common house fly (*Musca domestica*) and black soldier fly (*Hermetia illucens*) in poultry and fish diets. There is limited information available on the utilization of black soldier fly (BSF) as a feed ingredient in pig nutrition and no publications on its use in piglet diets. However, Newton *et al*. (1977) reported that similar production performances were achieved in adult pigs fed black soldier fly larvae meal (BSFLM) when compared to those fed soybean meal. Since it is a non-traditional feed and its use as an alternative protein source in piglet diets has not yet been investigated, there is the need for the study of its potential in the pig industry with particular attention to its effect on growth performance, health and manure microbiology. The hypotheses of this study were:

H_0: *Hermetia illucens* can be successfully utilized as an alternative protein source to partially replace other protein sources in the ability to sustain normal piglet performance; no differences expected.

H_1: *Hermetia illucens* cannot be successfully utilized as an alternative protein source to partially replace other protein sources in the ability to sustain normal piglet performance; differences expected.

The aims of this study were to investigate the effect of BSFLM as a protein source on piglet production. The specific objectives were to evaluate:

I. The production performance of piglets fed BSFLM.
II. The effect of BSFLM on blood haematological and biochemical parameters of piglets with respect to health aspects (immunology) and the concentration of minerals (bioavailability).
III. The effect of BSFLM on manure microbiology and manure texture.

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Chapter 2

Literature review

2.1 Introduction

The world is facing a drastic increase in the human population which has led to a considerable amount of pressure being placed on the agriculture sector to produce sufficient food (Dar and Gowda, 2013). This population increase has also led to the global animal production industry to suffer from a shortage in the supply of feed ingredients for animal use, which has led to suboptimal animal protein being produced for human consumption (Capper, 2013). This vicious circle has placed emphasis on the key term “sustainable”, which may be defined as “meeting the needs of the present without compromising the ability of future generations to meet their own needs” (Burton, 1987). Therefore, it has become increasingly important to find good-quality, renewable protein sources that are of at least equal in quality to already existing sources, which are able to substitute the traditional sources used in animal nutrition (Newton *et al*., 2005). This has presented the opportunity to explore into the broad spectrum of other possible sources of protein production for animal nutrition as these sources can be obtained from a variety of organisms, both flora and fauna (Newton *et al*., 1984; Pieterse, 2014).

In the production of food for human consumption, the agricultural sector produces an incredible tonnage of waste that is not utilized, but has the potential to be recovered and used in another sector (Cordell *et al*., 2009). Nature has provided for many methods of managing waste products of animal origin and these include bacteria, protozoa, fungi and insects (Bondari and Sheppard, 1987). Insects are also a valuable protein source for many animals, ranging over a wide variety of species (Newton *et al*., 1977; Bondari and Sheppard, 1987; Awoniyi *et al*., 2004). Therefore, nature has presented an effective, eco-friendly means of solving the problem of waste management and the opportunity to use these insects as a useful, sustainable protein source in animal diets (Bondari and Sheppard, 1987). Insect meals provide the essential amino acids for animal production and may minimize costs, which could consequently lead to the maximization of profits (Newton *et al*., 2005). There have already been reports of insect larvae meals being utilized as a renewable protein source in pig (Newton *et al*., 1977), poultry (Awoniyi *et al*., 2004) and fish (Bondari and Sheppard, 1987) nutrition. The larvae meal has resulted in good growth, development and production of these animals (El Boushy, 1991; Ijaiya and Eko, 2009; Hassan *et al*., 2009; Li *et al.*, 2011; Pieterse *et al*., 2014) and proved beneficial in certain environmental (El Boushy, 1991; Newton *et al*., 2005; Diener *et al*., 2009; Kim *et al*., 2011), health (Bondari and Sheppard, 1987; Pieterse, 2014; Pieterse *et al*., 2014; Pieterse *et al*., 2015) and economical (El Boushy, 1991; Newton *et al*., 2005) aspects. Thus, results acheived have led to insects being utilized as sustainable decomposers of organic waste (waste management potential) and as a useful nutrient recovery tool for its use in animal production. However, it has not yet been administered into the creep stage of pig diets. For the purpose of this review the process by which waste products are utilized by insects to produce a useful protein source shall be termed the ‘nutrient recirculation system’.

Due to the lack of studies carried out in pig production, there is very little acknowledgeable evidence that similar results, to those achieved in literature, would be discovered in creep stage trials.

However, regardless of species, the probability that similar results could be achieved cannot be disregarded, as the inclusion of fly larvae in pig diets would be partially replacing commercial protein sources with a source that would have already formed a part of the pigs' diet in the wild. This chapter therefore aims to review the various agricultural wastes that can be utilized as a larval feed substrate and the potential usage of larvae as an animal protein source**.** This study utilizes black soldier fly larvae meal (BSFLM) as a protein source in piglet production to investigate the effects of its inclusion on production, immunology (health) and manure bacterial shedding load. Thus, emphasis is placed on the black soldier fly (life cycle, organic waste decomposition and potential uses), digestibility and palatability of the larvae meal and the effect of its chemical components on pig blood and manure characteristics.

2.2 Organisms suitable for nutrient recirculation

There are many organisms which are suitable to be utilized in the nutrient recirculation system and there are three specific orders to take cognisance of, namely Diptera, Coleoptera and Haplotaxida (Bondari and Sheppard, 1987). The order of Diptera includes insects that are commonly known as true flies or two-winged flies. The insects that are familiar to this group include fruit flies, black soldier flies, house flies, midges and mosquitoes (Resh and Cardé, 2003). Insects that belong to this order are described as being ubiquitous, because they have the ability to colonize basically any habitat on earth (Scholtz and Holm, 1985; Resh and Cardé, 2003). Black soldier flies fall under the Stratiomyidae family, as the adults are found near larval habitats, which are found in a wide array of locations, mostly in wetlands, damp places in soil and under bark, in decaying organic matter and animal manure (Rozkošný, 1982). For the purpose of this literature review only insects from the Stratiomyidae family will be discussed further.

2.2.1 Stratiomyidae family

Hermetia illucens, the black soldier fly (BSF), is a common and widespread fly which belongs to the Stratiomyidae family and are non-biting flies, as they have no functioning mouthparts (Bondari and Sheppard, 1987). They can be found almost anywhere and have a tendency to gather around suitable breeding sites, which include garbage heaps, faeces (manure) and decaying matter. The larvae of these flies are also common detrivores[1] in compost heaps and are also found in association with carrion, where they have a significant potential for their use in forensic entomology (Lord *et al*., 1994). The black soldier fly has been considered to have great nutritional potential as a protein source in animal production, where it has already proved itself in studies undertaken in pig (Newton *et al*., 1977; Newton *et al*., 2005), poultry (Hale, 1973; Pieterse *et al*., 2014) and fish (St-Hilaire *et al.*, 2007) production.

[1] A detrivore is an organism that feeds on small pieces of decomposing plants and animals.

2.2.1.1 Description and life cycle

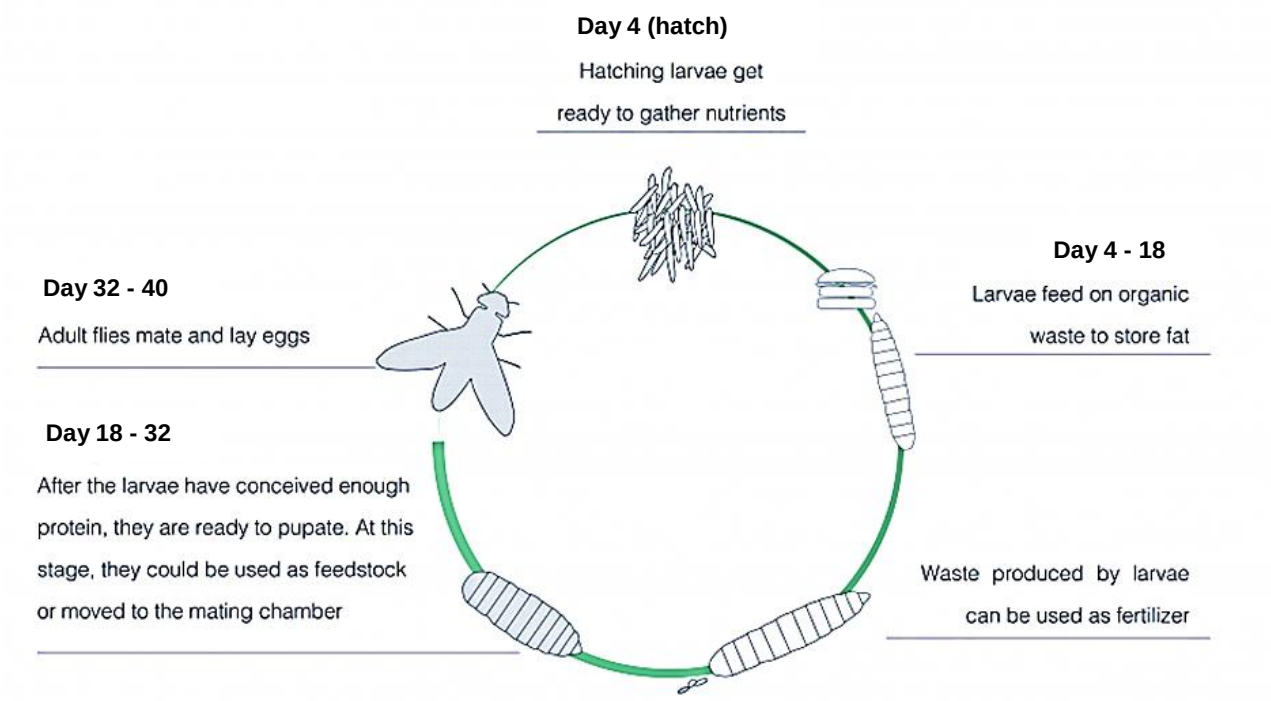

Figure 2:1 Life cycle of the black soldier fly, *Hermetia illucens*. (Adapted from Fok, 2014).

Figure 2:1 provides a visual of the life cycle of black soldier flies, which is approximately 40 to 44 days (Fok, 2014). A female produces a mass of eggs, ranging from 350 to 700, in her short life of five to eight days. By contrast, the house fly (*Musca domestica*) lives up to 30 days and during this period they must eat, and in doing so they are actively engaged in the spread of disease between animals (Sheppard *et al*., 2002). The fact that BSFs do not eat in their short life span as an adult, and therefore do not sit on food/waste, is the critical reason why they are not vectors of human pathogens (Bondari and Sheppard, 1987). The eggs of the BSF are typically deposited on surfaces in close proximity to decaying matter, such as compost or manure and take approximately four days to hatch (Sheppard, 1992). The newly hatched larvae are of light cream colour and crawl onto the waste, where they are able to consume at an amazing speed. Under ideal conditions it takes the larvae about two weeks to reach maturity, however, if the temperature is not ideal and if there is not enough food, then this period of two weeks may extend to several months (Sheppard *et al*., 2002). The ability of the BSF larvae to extend its life cycle when it is under conditions of stress is an extremely important reason why it may be used for waste disposal processing (Sheppard, 1992). Black soldier fly larvae pass through four stages, namely the egg, larvae (five instars), pupae and adult stage (Hall and Gerhardt, 2002). At maturity they are about 25 mm in length, 6 mm in diameter and weigh approximately 0.2 g. These larvae and pupae are very tough and robust and can survive under conditions of extreme oxygen deprivation (Sheppard *et al*., 2002).

The black soldier fly is a mimic, as it is very close in size, colour and appearance to the organ pipe mud dauber wasp (*Trypoxylon politum*) and its relatives (Sheppard *et al*., 2002). The mimicry to this particular kind of wasp is so enhanced that the fly's antennae are long and wasp-like, its hind tarsi is pale and the fly has two transparent 'windows' in the basal abdominal segments that make the fly appear to have a narrow wasp-like waist (Sheppard *et al*., 2002). This mimicry, acts as a predator

repellent which provides the fly with enough time to breed in its short life span (Sheppard *et al*., 2002).

2.2.1.2 Benefits of the black soldier fly

Black soldier flies have proved themselves to be extremely necessary and even beneficial in a number of biological processes that have been discovered to aid in animal production systems. They aid in controlling other insect pests, form part of the food chain and act as pollinators (second, right behind bees, as the main pollinators of plants), recyclers and scavengers (Sheppard *et al*., 2002). Black soldier fly larvae and fully grown flies are considered to be beneficial in the following ways:

I. They prevent houseflies and blow flies from laying eggs in the material that is inhabited by black soldier fly larvae (Sheppard *et al*., 2002).
II. They are not attracted to human habitation or foods, as they are detrivores and the egg bearing females are attracted to manure and decomposing material (Sheppard *et al*., 2002).
III. *Hermetia illucens* are not considered pests like that of other fly species, as they do not fly around as much as house flies and they are sanitary and do not bite or sting. It is easy to reduce their numbers by killing the pupae/larvae in a wet grub bin, before they become flies (Bondari and Sheppard, 1987).
IV. Unlike other fly species, *H. illucens* is not a disease vector and the larvae have resulted in significant reductions of *Escherichia coli* and *Salmonella enterica* in manure by modifying the microflora (Bondari and Sheppard, 1987).
V. Black soldier fly larvae reclaim would-be pollutants, where nine organic chemical odours are greatly reduced or even eliminated from manure in 24 h (Bondari and Sheppard, 1987).
VI. They quickly reduce the volume and weight of would-be waste, where the larvae breaks down its food and converts it to a valuable nutritional source, carbon dioxide respired by the grubs and symbiotic microorganisms (Sheppard *et al*., 2002).

2.2.1.3 Chemical composition of *H. illucens* larvae

Factors effecting chemical composition

Literature on the chemical composition of insect larvae meal and its suitability as a protein source varies throughout the publications available and these differences are related to the differences in the species, age at harvest (Calvert and Martin, 1969; Inaoka *et al*., 1999; Newton *et al*., 2005; Aniebo *et al*., 2008), method of drying (Fasakin *et al*., 2003) and larval feed substrate (Newton *et al*., 1977; Pieterse, 2014).

Fasakin *et al.* (2003) believed that the processing method would have an influence on the chemical composition of the larvae meal (Table 2:1), where the authors attributed this to the dilution effect of either the water or the fat on the remaining nutrients. However, the processing of raw materials is important as it allows for processors to adjust the chemical composition of the materials in order to make them more suitable for different species and different production stages of livestock. It can be seen in the table that the various methods of processing the fly larvae meal caused the crude protein content to vary between 43.30% and 46.70% on a dry matter basis. The crude protein content of the defatted larvae meal showing a tendency to increase with a decrease in the crude fat content of the meal. This can be explained, as when more oil is extracted from the meal, the protein content relative to the fat content increases (Shiau *et al*., 1990).

Table 2:1 Averages (± Standard error) of the moisture, crude protein, crude fat and ash of housefly larvae meal as influenced by processing methods (Fasakin *et al*., 2003).

Type of Larvae meal	Moisture (%)	Crude Protein (%)	Crude fat (%)	Ash (%)
Hydrolysed oven-dried	8.06 ± 0.05	45.60 ± 0.02	13.28 ± 0.03	13.20 ± 0.02
Hydrolysed sun-dried	8.40 ± 0.01	44.30 ± 0.03	13.65 ± 0.01	13.25 ± 0.01
Hydrolysed/defatted oven-dried	7.56 ± 0.02	46.70 ± 0.01	6.28 ± 0.01	13.30 ± 0.01
Hydrolysed/defatted sun-dried	8.10 ± 0.01	45.65 ± 0.01	6.30 ± 0.01	12.32 ± 0.02
Defatted oven-dried	9.20 ± 0.01	45.75 ± 0.03	7.00 ± 0.02	13.35 ± 0.02
Defatted sun-dried	9.65 ± 0.04	45.10 ± 0.05	7.40 ± 0.01	13.45 ± 0.02
Full fat oven-dried	8.25 ± 0.02	43.45 ± 0.03	14.30 ± 0.03	14.35 ± 0.02
Full fat sun-dried	8.55 ± 0.04	43.30 ± 0.01	14.35 ± 0.03	14.65 ± 0.01

Furthermore, Aniebo and Owen (2010) showed that the nutritional value of fly larvae meal is also significantly influenced by, not only the method in which the larvae are dried, but also the age at which the larvae are harvested (Table 2:2); the protein content of the larvae decreases ($P<0.05$) with age. The authors observed a decrease in the protein content, from 59.6 to 54.2 to 50.8% dry matter (DM), and an increase in the fat content, from 22.4 to 23.9 to 27.3% DM, when the larvae were dried at two, three and four days of age respectively. Reasons for this are related to the fact that as the larvae approach the pupae phase in metamorphosis they start to store more energy in the form of lipids (Pearincott, 1960) and that the larvae utilize the proteins in enzymatic reactions in the formation of the chitin layer (Kramer and Koga, 1986). Aniebo and Owen (2010) also reported that sun drying produced larvae with a lower protein content and a higher fat content when compared to oven dried larvae.

Table 2:2 Average (± standard error) crude protein and fat content (DM basis) of house fly larvae as affected by age and method of drying (Aniebo and Owen, 2010).

	Day 2 Harvested	Day 3 Harvested	Day 4 Harvested
Oven Dried			
Crude Protein	59.6^{a} ± 0.05	54.2^{b} ± 0.03	50.8^{a} ± 0.04
Fat	22.4^{a} ± 0.14	23.9^{b} ± 0.14	27.3^{c} ± 0.35
Sun Dried			
Crude Protein	55.3^{a} ± 0.14	51.3^{b} ± 0.04	45.5^{c} ± 0.74
Fat	25.2^{a} ± 0.14	28.0^{b} ± 0.14	32.0^{a} ± 0.35

$^{a-c}$ Means within a row with different superscripts differ ($P<0.05$).

Chemical composition of H. illucens in literature

The proximate analysis of any nutritional source provides valuable information in regards to the moisture, protein, fat, fibre, mineral and energy content of different ingredients and/or diets. The values of these respective components provide the base in which diets are formulated to meet the animals' requirements for different productive stages. Table 2:3 shows the variation in the proximate analysis values of BSF larvae and prepupae as achieved by the various authors.

Table 2:3 Comparison of black soldier fly larvae and prepupae composition (DM basis) receiving different feed substrates.

	FAO, 2015	Newton *et al*., 2005		St-Hilaire *et al*., 2007	Pieterse, 2014
Feed Substrate		Pig manure	Poultry Manure	Pig Manure	Kitchen/Food Waste
Stage at Harvest		Prepapue	Prepupae	Prepupae	Larvae
Dry matter (% as fed)	91.30	-	-	91.60	91.30
Crude protein (%)	42.10	43.20	42.10	43.60	30.63
Crude fibre (%)	7.00	-	7.00	-	11.11
Ether extract (%)	26.00	28.00	34.80	33.10	44.72
Ash (%)	20.60	16.60	14.60	15.50	8.60
Gross energy (MJ/kg)	22.10	-	-	-	22.10

Table 2:4 presents the mineral composition of larvae and prepupae meal as reported by the various authors. The differences seen among them are attributed to the differences in the feed substrates, stage of harvest (larvae vs. pupae) and processing methods used in animal nutrition. In this table it is indicated that the prepupae, if fed different feed substrates, will have some mineral content variations, but the values that are of some significance are those that were fed poultry manure that showed a much higher iron value (137.0 vs. 77.6 mg/100 g). Fasakin *et al*. (2003) also discovered that processing had an effect on the mineral content of the larvae meal and their findings showed that the process of hydrolysis and defatting of the larvae meal caused an increase in the levels of calcium, magnesium and manganese. This, as mentioned previously, is due to the fact that the extraction of the oil caused the amount of feed product to decrease, concentrating the specific nutrient (minerals). Thus, the relative values of all the minerals increased in comparison to each other. Differences in the mineral content of BSFs reared on poultry and pig manure also reflect a variation in the feed substrates, where phosphorus was significantly higher in prepupae reared on poultry manure (Newton *et al*., 2005).

Table 2:4 Mineral compositions of processed black soldier fly larvae and prepupae.

Minerals Analysed	**FAO, 2015**	**Newton *et al*., 2005**		**Pieterse, 2014**
Feed Substrate		Pig Manure	Poultry Manure	Kitchen/Food Waste
Stage at Harvest		Prepupae	Prepupae	Larvae
Processing Method		Dried at 70°C	Dried at 70°C	Dried at 60°C
Calcium (g/100g)	7.56	5.36	5.00	5.00
Phosphorus (g/100g)	0.90	0.88	1.51	0.64
Potassium (g/100g)	0.69	1.16	0.69	0.69
Sodium (g/100g)	0.13	0.13	0.13	0.13
Magnesium (g/100g)	0.39	0.44	0.39	0.39
Manganese (mg/100g)	24.60	34.80	24.60	-
Zinc (mg/100g)	10.80	27.10	10.80	-
Copper (mg/100g)	0.60	2.60	0.60	-
Iron (mg/100g)	137.00	77.60	137.00	-

The amino acid profiles reported vary greatly between the various authors (Table 2:5) which, despite the difference in feed substrate, stage at harvest and processing method, is also attributed to the different laboratory techniques undertaken by the respective authors when analysing the amino acid

profile of the fly larvae. Newton *et al*. (2005) and Pieterse (2014), both hydrolysed their samples before analysis, however, Newton *et al*. (2005) utilized a Durrum Model D-500 amino acid analyser and Pieterse (2014) determined the specific amino acid content through precolumn derivatisation, which separated them using high performance liquid chromatography. This procedure was completed by the detection of the amino acids using a fluorescence detector. St-Hilaire *et al.* (2007) conducted the amino acid analysis utilizing the A.O.A.C.-approved method described at http://www.eurofinsus.com/Item.html_itemServices/Services%20(2005-01-01.html (Eurofins US, Petaluma, CA, USA). It is noted from the literature that Newton *et al*. (2005) were the only authors that tested for and reported on traces of tryptophan in their analysis, where they utilized the procedure of Amaya-F *et al.* (1976) which has a high recovery rate for this specific amino acid. Other methods undertaken by the other authors represented in Table 2:5 did not allow for tryptophan detection, as there is a high cost associated with such a test.

Table 2:5 Amino acid profile of black soldier larvae and prepupae (g/100g DM) receiving different feed substrates.

Amino Acid	**FAO, 2015**	**Newton *et al*., 2005**		**St-Hilaire *et al*., 2007**	**Pieterse, 2014**
Feed Substrate		Beef manure	Pig manure	Pig manure	Kitchen/Food Waste
Stage at Harvest		Prepupae	Prepupae	Prepupae	Larvae
Processing method		Dried at 70°C	Dried at 70°C	Dried at 80^{0}C	Dried at 60°C
Alanine	3.24	3.69	2.55	2.45	2.05
Arginine	2.36	2.24	1.77	1.78	2.38
Aspartic acid	4.63	4.56	3.04	4.09	3.19
Cystine	0.04	0.06	0.31	-	-
Glutamic acid	4.59	3.81	3.99	4.42	3.74
Glycine	2.40	2.88	2.07	1.72	1.99
Histidine	1.26	1.91	0.96	0.76	1.23
Isoleucine	2.15	1.96	1.51	1.83	1.62
Leucine	3.33	3.53	2.61	2.66	2.48
Lysine	2.78	3.37	2.21	2.05	2.41
Methionine	0.88	0.86	0.83	0.77	0.69
Phenylalanine	2.19	2.20	1.49	1.83	1.52
Proline	2.78	3.26	2.12	-	1.91
Serine	1.31	0.12	1.47	1.37	1.62
Threonine	1.56	0.55	1.41	1.58	1.51
Tryptophan	0.21	0.20	0.59	-	-
Tyrosine	2.90	2.51	2.38	2.22	2.27
Valine	3.45	3.41	2.23	2.99	2.03

Table 2:6 shows the calculated ratio of essential amino acids to that of lysine. In pigs, lysine is regarded as the first limiting amino acid and supplementing this amino acid to deficient diets increases the efficiency of protein utilization (PIC, 2008). In the ideal amino acid profile for any animal, all the essential amino acids are expressed as a percentage of lysine because the essential amino acids relative to lysine remains constant regardless of genetic, dietary and environmental factors (Schutte and de Jong, 2004). Newton *et al.* (2005) and Pieterse (2014) reported results that had the closest amino acid to lysine ratios when compared to the ideal amino acid profile for pigs,

with slight variations between them. These variations can be attributed to the different processing methods and feed substrates used by the authors. This indicates the importance of constant amino acid analysis regarding the different rearing conditions and methods of processing BSFLM. The importance of interactions is also noted, as other protein sources, as well as the other components of the diet, must be fed in conjunction with the fly larvae meal in order to achieve the best amino acid profile for the animal.

Table 2:6 Calculated amino acid to lysine ratios of black soldier fly larvae and prepupae meal in comparison to the ideal amino acid profile for nursery (3.6 – 22.7 kg) pigs.

Amino Acid	**FAO, 2015**	**Newton *et al*., 2005**		**St-Hilaire *et al*., 2007**	**Pieterse, 2014**	**Ideal Amino Acid profile***
Feed Substrate Stage at harvest		Beef manure Prepupae	Pig manure Prepupae	Pig manure Prepupae	Kitchen/Food Waste Larvae	
Lysine	100	100	100	100	100	100
Methionine + Cystine	33	27	52	-	-	58
Threonine	56	16	64	77	67	60
Tryptophan	8	6	27	-	-	17
Isoleucine	77	58	68	89	70	55
Valine	124	101	101	146	86	65

*Ideal amino acid profile as according to the PIC Nutritional Specifications 2008.

Table 2:7 presents the fatty acid composition of BSF larvae on an as is basis and as processed prepupae, as reported by the different authors, and it shows that there was considerable variation between them. On an as is basis the contents of the larvae was significantly higher in regards to myristic acid and linoleic acid than the processed prepupae. This explains the possible effect that stage at harvest and/or processing method has on specific fatty acid components of the end meal product.

Table 2:7 Fatty acid composition of black soldier fly larvae on an as is basis and as processed prepupae, expressed as a percentage (%).

Fatty Acid	**St-Hilaire *et al*., 2007**	**Sealey *et al*., 2011**		**Finke, 2013**
Feed Substrate	Pig Manure	Cattle Manure	Fish Offal	Did not Specify
Stage at Harvest	Prepupae	Prepupae	Prepupae	larvae
Processing method	Dried at 80°C	Dried at 40°C	Dried at 40°C	As Is Basis
Capic 10:0	-	-	-	0.69
Lauric 12:0	49.34	23.60	37.10	51.20
Myristic 14:0	6.83	5.10	6.30	12.00
Myristoleic 14:1	-	-	-	0.50
Pentadecanoic 15:0	-	-	-	0.12
Plamitic 16:0	10.48	19.80	17.30	16.10
Palmitoleic 16:1	3.45	6.30	7.60	4.96
Heptadecanoic 17:0	-	-	-	0.20
Heptadecenoic 17:1	-	-	-	<0.08
Stearic 18:0	2.78	6.50	2.00	2.45
Oleic 18:1	11.81	22.70	18.80	15.60
Linoleic 18:2	3.68	6.80	5.90	16.90
Linolenic 18:3	0.08	0.00	0.50	0.65
Arachidic 20:0	-	-	-	0.16
Eicosenoic 20:1	-	-	-	<0.08
Eicosadienoic 20:2	-	-	-	<0.10
Arachidonic 20:4	-	-	-	<0.08
Benhenic 22:0	-	-	-	0.09

2.3 Feed substrates/ waste products

If the focus is specifically on South Africa alone, there are many different sources of organic waste of which most can pose as a health risk if not managed properly (Roberts and de Jager, 2004). The nutrient recirculation organisms can potentially reduce the build-up of these waste products, especially from the agricultural sector, as they are able to utilize these products as a food source. The waste products from the agricultural sector include that from abattoirs, food retailers and the fermentation industry.

2.3.1 Waste from agriculture

The agricultural sector has various direct waste products which include manure, harvest residues and waste from processing plants (blood, whey, rejected food, etc.). This waste is usually turned into compost and used as fertilizers, however with the increasing concern for sustainable fuels, there is an increasing use of waste material for the production of biogas (Abraham *et al*., 2007). Manure can serve as a potential source of nutrients for BSFs and there have been reports on the efficiency of which this waste can be converted to a valuable protein source (Calvert and Martin, 1969; Newton *et al*., 2005; St-Hilaire *et al*., 2007; Sealey *et al*., 2011; Pieterse *et al*., 2015). El Boushy (1991) reported that there are various factors that have a significant influence on the chemical composition of manure, these include animal species, age, feeding ration and the amount of undigested feed present in the manure. Storage time of manure also has an influence on the chemical composition,

where a reduction in the crude protein content is expected with an increase in the storage time (Flegal *et al*., 1972). These factors may have an influence on the effective conversion of these would be wastes by the BSF larvae, which in turn may affect the nutritional composition of the larval end product. Thus, it would be necessary to look into possible methods of nutrient binding/capturing to limit these nutrient losses with the increase in storage time (Flegal *et al*., 1972).

Secondary waste products of the agricultural sector are those that come from the commercial industry, which includes that from abattoirs, food retailers and the fermentation industry.

2.3.1.1 Waste from abattoirs

Abattoir waste includes the blood, bones, intestines, intestinal contents, carcass trimmings, heads, hooves/feet, hides, dead on arrivals, rejected carcasses, feathers and fat of animals (Roberts and de Jager, 2004). However, in South Africa there is a market for particular parts of the animal, which is known as offal[2]. Hooves are utilized in the glue industry and feathers can either be utilized in the household goods industry for the manufacturing of pillows and duvets or they are used in the animal feed industry as a protein source (Dalev, 1994).

Blood from the abattoirs can be utilized in the manufacturing of blood meal, which is a very rich source of protein, at approximately 889 g/kg DM (NRC, 1994). It has a good amino acid profile, but taking into the concept of certain health risks, its use is either banned completely or restricted to an absolute minimum as an animal feed in many countries over the world. The rejected animals and the dead on arrivals can be utilized in the manufacturing of carcass meal for its use in the animal feed industry, but has also been banned or restricted in most countries. In South Africa the use of blood and carcass meals as an animal feed has not been banned, however, the use of certain meals has legally been deemed as an unacceptable practise (Act No 36 of 1947). In Africa and many other countries, any animal product that can be a source of Bovine Spongiform Encephalopathy (BSE) are unacceptable as a source of feed to animals in terms of the Codex Alimentarius Commission (CAC/RCP 54-2004).

There are various methods in which abattoir waste can be disposed of and these include that by the municipal/local authority drainage, oxidation dams, run-off into the fields whilst rejected carcasses can be placed into a trench dug into the ground, to undergo decomposition (Roberts and de Jager, 2004). However, there are numerous health and environmental risks with these practises, as ground water supplies may be contaminated or polluted with pathogens, also affecting those animals that depend on it to survive (Mittal, 2006).

The largest volume of abattoir waste is presented by the blood and intestinal content and the rejected carcasses (Christoe, 2003), thus, the emphasis here is that these waste products can be utilized by the dipteran larvae for nutrient recirculation. Therefore, the recirculation process can have a positive impact on the environment, by reducing the risk of contamination, and on the animal feed industry as a valuable protein source is being created from waste. This subject has received much attention (Aniebo *et al*., 2008; Aniebo and Owen, 2010) and to place it into perspective: Rainbow chickens is the largest producer of broilers in South Africa, slaughtering 4 million birds per week (http://www.rainbowchickens.co.za/about). If a broiler has an approximate dressing percentage of 70%, therefore losing up to 30% of their total body weight as waste (Haitook, 2006), then 4 million broilers weighing ~1.9 kg each will produce up to 2280 tonnes of waste per week.

[2] Offal are the entrails and internal organs of an animal used as food.

Thus, when considering mass production, there is a considerable amount of waste that just is available for nutrient recirculation. Thereby solving the problems of proper waste removal, environmental contamination, possible health risks associated with contamination and acquiring an alternative, feasible protein source for animal production.

2.3.1.2 Waste from the fermentation industry

The fermentation industry includes the sectors of brewery, distillery and meat and milk processing factories. The by-products associated with the brewing industry include brewer's grain, spent hops, malt culms and brewer's yeast (McDonald, 2002). Brewer's grain is a good source of digestible fibre, has a high protein content (approximately 24.2% DM) and is high in phosphorus, but low in the other minerals such as calcium and potassium and it is usually fed to ruminants, pregnant sows and growing pigs (Santos *et al*., 2003). Spent hops is high in fibre, but rarely used in the animal feed industry and mostly sold to be utilized as fertilizer (Huszcza and Bartmanska, 2008). Malt culms is rich in protein (approximately 375 g/kg DM) and a fibrous type of feed, but low in terms of energy (Brouns *et al*., 1995). Brewer's yeast is a by-product that is rich in protein (approximately 420 g/kg DM) and a valuable source of B vitamins (except vitamin B_{12}) and phosphorus. It is highly digestible with a relatively high nutritional value and has reasonably low calcium content, but is favoured as a suitable feed by all classes of farm animals (McDonald, 2002).

The distilling industry includes by-products such as distiller's grain, distiller's grain with soluble (DGS), distiller's dark grain and also malt culms. The composition of distiller's grain varies, depending on the method of processing and the quality of the commodity before processing, but it is usually high in unsaturated fatty acids and fibre, but low in terms of dry matter content (McDonald, 2002). Distiller's grain with soluble is a valuable source of the B vitamins and protein (ranging from 23.4 to 28.7% DM), however within the feed industry, there is a high degree of variability in the nutritional properties of the DGS available (McDonald, 2002).

Whey is a by-product from the cheese making industry and its composition varies according to the type of cheese produced (Thivend, 1977). Whey has most of its protein in the form of β-lactoglobulin which is of very good quality and is usually fed to pigs in the liquid form or dried effectively and added to piglets' diets, however, is lacking with regards to energy, calcium, phosphorus and fat-soluble vitamins (McDonald, 2002).

2.3.1.3 Waste from retailers

Wastages from the retail industry includes consumer or food service losses from uneaten and damaged products, losses from the transportation of the food from the farm to the retailer and retail losses due to past due-date products (Kantor *et al*., 1997). Kantor *et al*. (1997) reported that the estimated food losses in America from retail stores to be 2.5 million tonnes of waste per annum, where less than 5% comes from edible material. Waste coming from the food service industry and households is estimated at approximately 42.3 million tonnes of which 26% comes from edible material and of that 20% is accounted for by fresh fruits and vegetables.

Table 2:8 shows the projected daily waste loading rates for *H. illucens* for various kinds of organic waste based on their energy values with the digestibility energy of chicken feed as the reference value (Diener *et al*., 2009). The values projected in the table are based on rough estimations and would require to be interpreted by experimental procedures, as they depend on other factors, such as material moisture or fibre content. Diener *et al*. (2009) reported that, depending on the larval

density and waste source, a daily loading rate of 3 to 8 kg per m^2 can be applied to the BSF larvae nutrient recirculation system. Pieterse *et al*. (2015) reported similar findings, where 10 kg of feed fed per m^2 per day would result in a 1 kg of wet larvae harvested per m^2 per day. Over the 14 day period (length of larval stage), 10 kg of feed fed per m^2 would result in the harvesting of 14 kg per m^2 or 1 kg per m^2 per day of the wet larvae.

Table 2:8 Projected daily waste loading rates for a black soldier fly larvae nutrient recirculation system based on the energy values of various organic waste sources[a] (Diener *et al*., 2009).

Feed substrate	Gross energy (MJ/kg)	Digestible energy (MJ/kg)	Daily feeding rate (mg/larvae/day)	Waste loading rate/m^2 (kg)[b]	Energy data source
Chicken feed[c]	-	11.7	100	5	
Kitchen waste	22	19.3[d]	61	3.05	(ALP. 2007)
Vegetable waste	17.6	11.9[d]	98	4.9	(ALP. 2007)
Pig manure	17.8	7.4	158	7.9	(Ulloa *et al*., 2004)
Poultry manure	14.6	6.7	175	8.75	(Ulloa *et al*., 2004)
Human faeces	21.6	-	130[e]	6.5	(Diem and Lentner., 1968)

[a]Calculation basis: 100 mg chicken feed per larvae per day (60% moisture).
[b]Assumption: 50 000 larvae m^2.
[c]UFA 625 Alleinfutter fur Legehennen UNIVERSAL.
[d]Digestible energy for pigs.
[e]Calculation assumed the same gross energy/digestible energy ratio as for pig manure.

2.4 The utilization of black soldier fly larvae meal in animal nutrition

Linder (1919) was the first to report on the production of insects as a protein source for animal production from waste products. This study dates back to nearly a century ago, where the use of multicellular organisms to convert waste to useful products was undertaken. In Linder's study, common house fly larvae were reared on sewage and then harvested, dried out and fed to caged rats. However, this project did not progress very far (incomplete) and the next publication of interest was the work done by Calvert and Martin (1969), where they studied the use of the common house fly (insect meal) to produce valuable nutrients from poultry waste. Their study led to the conclusion that dried housefly pupae could provide sufficient protein for normal growth and development of broilers during the early stages of their life.

In South Africa, but also around the world, there is an on-going concern towards the ever-increasing feed prices, especially for protein sources such as fish meal, and this has placed more emphasis for research to be conducted into alternative nutritional sources. In most literature published to this date, the use of fly larvae meal has compared favourably with other protein sources commonly used in animal feeds. Newton *et al*. (1977), Newton *et al.* (2005), St-Hilaire *et al*. (2007), Sealey *et al*. (2011) and Finke *et al*. (2013) concluded in their studies that BSFLM has a suitable nutritional composition and can serve as an alternative (partial replacement) for fish meal, as well as other protein sources used in animal nutrition.

Table 2:9 gives a comparison of BSF larvae to fish meal, soya oilcake meal and sunflower oilcake meal. It can be seen from this table that the larvae meal is superior, with regards to certain composition traits, to some of these traditional protein sources; however in other traits it is inferior. Black soldier fly larvae meal has a relatively high crude protein and gross energy content and in

comparison to the other protein sources has a significantly higher ether extract and ash content. The larvae meal has a significantly superior mineral content with regards to calcium, manganese and iron, but has a relatively low potassium content when compared with the other sources. Newton *et al*. (2005) reported that larvae meal produced on pig manure should be similar to soya oilcake meal in lysine, leucine, phenylalanine and threonine; higher in methionine histidine, valine and tryptophan; and lower in in isloleucine and arginine. It can be seen in Table 2:9 that the amino acid content of the BSFLM does follow the desired comparative composition, besides that of tryptophan and isoleucine. This may be explained by the use of different feed substrates, as it was not specified by the Food and Agriculture Organization (FAO), due to its values being the representable average of various authors' publications. Further, the amino acid profile of BSF prepupae may be improved by additional fractionation to remove the chitinous cuticle, which represents a small, but acknowledgeable portion of the protein content (Newton *et al.*, 2005).

Table 2:9 Comparison between the nutritional composition of black soldier fly larvae meal and other traditionally used protein sources (FAO, 2004).

		Black soldier fly larvae meal	Fish meal	Soya oilcake meal	Sunflower Oilcake meal
Main analysis	**Unit**				
Dry matter	% as fed	91.3	92.1	87.9	89
Crude protein	% DM	42.1	75.4	51.8	32.4
Crude fibre	% DM	7	-	6.7	27.9
Ether extract	% DM	26	11	2	2.2
Ash	% DM	20.6	13.6	7.1	7.1
Gross energy	MJ/kg DM	22.1	21.9	19.7	19.4
Minerals					
Calcium	g/kg DM	75.6	26.5	3.9	4.4
Phosphorus	g/kg DM	9	22.3	6.9	11.6
Potassium	g/kg DM	6.9	11.9	23.7	16.9
Sodium	g/kg DM	1.3	10.9	0.1	0.1
Magnesium	g/kg DM	3.9	3.1	3.1	5.6
Manganese	mg/kg DM	246	10	45	38
Zinc	mg/kg DM	108	99	54	96
Copper	mg/kg DM	6	-	18	32
Iron	mg/kg DM	1370	-	346	271
Amino acids					
Lysine	% DM	2.8	5.7	3.2	1.1
	% lysine	100	100	100	100
Alanine	% lysine	116.7	81.3	72.1	122.9
Arginine	% lysine	84.8	77.3	121.3	231.4
Aspartic acid	% lysine	166.7	116.0	185.2	251.4
Cystine	% lysine	1.5	10.7	24.6	48.6
Glutamic acid	% lysine	165.2	168.0	290.2	540.0
Histidine	% lysine	45.5	29.3	42.6	68.6
Isoleucine	% lysine	77.3	57.3	75.4	117.1
Leucine	% lysine	119.7	93.3	123.0	177.1
Methionine	% lysine	31.8	37.3	23.0	65.7
Phenylalanine	% lysine	78.8	50.7	82.0	125.7
Serine	% lysine	47.0	53.3	82.0	120.0
Threonine	% lysine	56.1	54.7	63.9	102.9
Tryptophan	% lysine	7.6	14.7	21.3	37.1
Tyrosine	% lysine	104.5	38.7	57.4	65.7

Although all these meals prove to be excellent sources of protein, there are still acknowledgeable differences which separate them from one another. Table 2:9 provides a clear indication as to how these respective sources differ in their nutritive value and as to how these protein sources can be utilized together to complement each other in the animal feed industry. Interactions between these different sources can be exploited to provide animals with the correctly balanced amino acid profile for optimal production.

2.4.1 Pig nutrition

Protein is quantitatively one of the most expensive nutrients in pig diets and the urgency of research to be conducted into alternative sources is ever increasing (Newton *et al*., 2005). Pigs require a good source of protein for proper growth and development, most importantly for the development of muscle tissue (Van Heugten, 2010). Protein is made up of amino acids, which contains nitrogen and this is what distinguishes protein from other feed components, such as fat and carbohydrates (Reece *et al*., 1995). The amino acids and their balance with each other is incredibly important in animal, particularly monogastric, nutrition as the supply of essential amino acids[3] is critical for pig production (Goodband *et al*., 2014). Lysine is the first limiting amino acid, this implies that without sufficient quantities of it the other amino acids cannot combine properly to form muscle protein (Reece *et al*., 1995; Goodband *et al*., 2014). However, emphasis is also placed on the importance of the correct supply of the other feed components (fat, energy, minerals, and fibre) of the diet, as the full potential of each of these components to be properly absorbed and utilized is correlated with one another (Reece *et al*., 1995). The correct balance of energy to amino acids ensures good muscle development, but an oversupply of energy would lead to excess fat deposition and a consequent decrease in market price. Whereas excess protein (specifically lysine) would be broken down into non-essential amino acids or would be lost in the urine, making it costly (Reece *et al*., 1995). Furthermore, a detrimental effect on growth and feed efficiency may also be associated with too much lysine. Reece *et al*. (1995) describes many factors that are considered in diet recommendations and formulations (Figure 2:2).

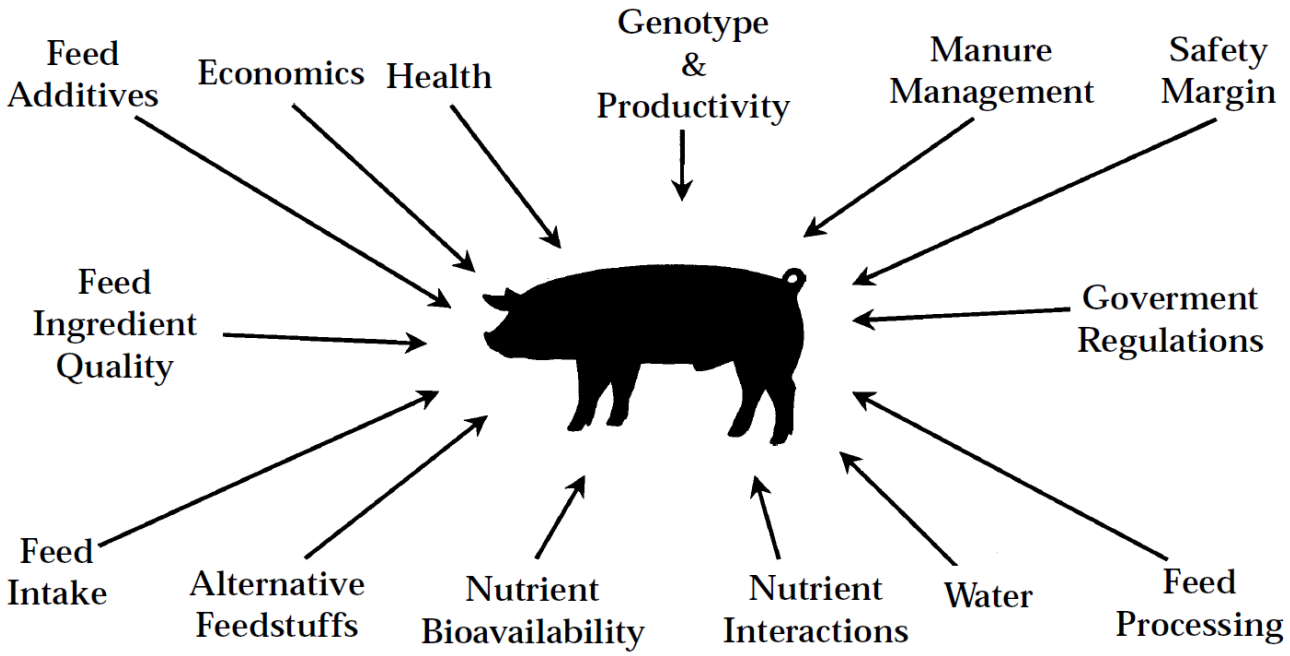

Figure 2:2 Factors that are considered when developing pig nutrient recommendations (Reese *et al*., 1995).

Pig producers are faced with the constant challenge to supply a correctly balanced diet, as pigs are expected to grow to market weight in the shortest possible time without sacrificing good carcass characteristics, so as to maximize profits (Van Heugten, 2010). Black soldier fly larvae appears to be a sustainable and alternative protein source to achieve such a balance for optimal pig production as discussed in the chemical composition of this chapter. However, there is very little literature on

[3] Essential amino acids are those that must supplied by the feed, as the animal is unable to synthesise them.

the use of larvae meal in the diets of pigs, specifically in the diets of piglets, and the only real publications of interest was the work by Newton *et al*. (1977), where they investigated the utilization of dried *H. illucens* larvae meal as a supplement for pigs. Additional literature published by Newton *et al*. (2005) on the utilization of black soldier fly larvae as a value-added tool for the management of pig manure.

2.4.1.1 Digestibility of black soldier fly larvae meal

Newton *et al*. (1977) performed a digestibility trial, where fly larvae were grown on cattle manure and urine slurry and then processed by means of washing, oven drying (85°C) and grinding by hammer mill. After grinding, solvents (35 mg/kg of butylated hydroxyl anisole and 35 mg/kg butylated hydroxyl toluene) were added to the larvae meal to stabilize the lipids and the meal was frozen until diet formulation. Proximate analysis results showed that the larvae contained 42% crude protein, 35% ether extract and 5% calcium. Two diets were formulated to contain 20% crude protein and 13% ether extract utilizing either the fly larvae meal or soybean meal with added stabilized brown grease (Table 2:10).

Table 2:10 Ingredient composition of the digestibility trial diets (Newton *et al*., 1977).

	Diets	
Item (%)	**Soybean meal**	**Larvae meal**
Dried *Hermetia illucens*	-	33
Soybean meal	25.5	-
Yellow maize	61.05	63.55
Stabilized brown grease	10	-
Calcium phosphate	1.5	1.5
Limestone	0.7	0.7
Sodium Chloride[a]	0.5	0.5
Vitamin premix[b]	0.5	0.5
Antibiotic premix[c]	0.25	0.25

[a]Minimum guarantee - Iron 1.0%; Zinc 0.4%; Manganese 0.2%; Copper 0.1%; Cobalt 0.01%; Iodine 0.01%.

[b]Minimum guarantee/kg - Vitamin A 441,000 USP units; Vitamin D 882,000 USP units; Riboflavin 1,544 mg; D-pantothenic acid 4.410 mg; Niacin 6,615 mg; Choline chloride 66,150 mg; Vitamin B 8,8 mg; Vitamin E 2,205 IU and Zinc 20 g.

[c]To supply 44 mg Chlortetracycline/kg of diet.

Newton *et al*. (1977), fed these diets to six 5-week-old barrow pigs in a digestion trial using two pigs and two periods in each of the three Latin squares, so that each pig received both diets (2 × 2 Latin square design). These pigs were weaned at four weeks of age and fed a diet containing 6% added fat, where half of the supplementary protein was derived from soybean meal and the other half from meat and bone meal prior to the start of the trial. Animals were then offered the treatment diets; a soyabean meal based diet and larvae meal based diet. The trial was carried out for a period of 10 days and the pigs were able to feed from self-feeders once daily with those within a square being offered identical amounts of feed. The specific intake of each individual was then calculated and recorded. They were housed in individual cages which allowed for the separate collection of the faeces and urine which was analysed. A proximate analysis was performed, using A.O.A.C. (1970) methods, on the larvae meal, feed, feed refusal, faecal samples and urinary nitrogen and results as obtained by Newton *et al*. (1977) are presented in Table 2:11.

Table 2:11 Proximate composition and calcium and phosphorus content of dried larvae and digestion trial diets, as well as the intake and digestion coefficients of the respective diets (Newton *et al*., 1977).

		Diet	
Item (% DM)	**Dried larvae**	**Soybean meal**	**Larvae meal**
Crude protein	42.1	20.5	20.6
Ether extract	34.8	11.7	13.5
Crude fibre	7.0	2.1	3.8
Moisture	7.9	10.7	10.1
Nitrogen free extract	1.4	59.9	53.0
Ash	14.6	5.9	9.0
Calcium	5.0	0.9	2.7
Phosphorus	1.5	1.1	0.9
Intake and Digestion Coefficients[a]			
Dry matter			
Intake, g		518.5	492.0
Apparent absorption, %		85.3	77.5[b]
Ether extract			
Intake, g		59.2	72.1
Apparent absorption, %		73.0	83.6
Crude fibre			
Intake, g		10.1	18.4[b]
Apparent absorption, %		49.2	53.8
Ash			
Intake, g		31.0	44.4[c]
Apparent absorption, %		61.7	45.2[b]
NFE			
Intake, g		311.4	252.6
Apparent absorption, %		91.2	84.7[b]

[a]Mean of 6 observations per treatment.

[b]P<0.05

[c]P<0.06

From Table 2:11 it can be seen that the dried larvae had a high content of calcium and this could be explained by the fact that the hypodermis of *H. illucens* secretes a deposit of calcium carbonate (Johannsen *et al*., 1922). On analysis, the soybean meal diet contained less ether extract than the larvae meal diet despite the fact that both the diets were formulated to contain equal amounts. The soybean meal diet also showed somewhat higher traces of calcium and phosphorus, although it was calculated to provide NRC (1973) recommended levels. Apparent digestibility of dry matter for the pigs fed the larvae meal diet was significantly lower than for those that were fed the soybean meal diet and these differences can be accounted for by the differences in the apparent digestibilities of ash and NFE between the two diets, but as to what extent is unknown. Published literature by Brooks (1967) explains that by increasing the dietary fat by as much as 10% could result in only slight and variable effects on dry matter digestibilities. The ash intake was highest (P<0.06) for the pigs fed the larvae meal diet and was incompletely characterized, therefore, it may have contained a component that reduces digestibility, as has been reported to be the case with silica (Smith *et al*., 1973). The digestibility of ether extract was greatest for the larvae meal diet, even though its intake was greater

when compared to that of the soybean meal diet and its faecal output was lower. This phenomenon is explained by the fact that the major food reserves of insects are triglycerides and that black soldier fly larvae store large quantities of this fat as an energy source to carry them through pupation (Chapman, 1971).

Table 2:12 presents the digestibility results for nitrogen, calcium and phosphorus as achieved by Newton *et al*. (1977) and although the difference in apparent nitrogen digestibility was slight ($P<0.05$), it was significantly in the favour of the soybean meal diet. The urinary nitrogen excretion was higher for the pigs fed the larvae meal diet, which along with a lower intake, resulted in a lower nitrogen balance when compared to those pigs fed the soybean meal diet. When expressed as a percentage of intake, this effect on nitrogen balance was significant ($P<0.05$). This could be explained, as when the two diets were compared on an amino acid basis, it was discovered that both were low in regards to methionine and cystine and that, in addition, the larvae meal diet was low in terms of threonine and tryptophan for pigs in the weight class of those utilized in the trial (Newton *et al*., 1977). Thus, this imbalance in essential amino acids possibly accounted for the lower nitrogen retention in pigs fed the larvae meal diet.

Table 2:12 Nitrogen, calcium and phosphorus digestibility and balance for pigs fed soybean meal and fly larvae meal diets[a] (Newton *et al*., 1977).

Item	Diets	
	Soybean meal	**Larvae meal**
Nitrogen		
Intake, g	17.2	16.7
Faecal, g	3.8	3.8
Urinary, g	4.1	4.8
Apparent digestibility, %	77.2	76.0[b]
Balance, g	9.3	8.1
Balance, % of intake	53.3	47.2[b]
Calcium		
Intake, g	3.99	12.79
Faecal, % of intake	60.74	61.09
Urinary, % of intake	9.62	13.74
Apparent digestibility, % of intake	39.26	38.91
Balance, % of intake	29.64	25.16
Phosphorus		
Intake, g	5.47	4.24[c]
Faecal, % of intake	48.74	75.51[c]
Urinary, % of intake	2.34	1.48
Apparent digestibility, % of intake	51.26	24.49[c]
Balance, % of intake	48.92	23.01[c]

[a]Mean of 6 observations per treatment

[b]$P<0.05$

[c]$P<0.06$

A major component of the exoskeleton of an insect is chitin, which is a polymer of N-acetyl glucosamine and glucosamine, and represents a source of non-protein nitrogen in diets containing

insects (Chapman, 1971). During the larval stage of the *H. illucens* life cycle, the insects contain very small traces of chitin, if any, and although glucosamine was included in the amino acid analysis, none was detected in the larvae meal. However, only 98% of the total nitrogen was accounted for by the analysis, which would provide a theoretical indication that only a very small fraction of the total nitrogen might be unavailable to the pig (Newton *et al*., 1977). Šimůnek *et al*. (2001) reported that although pigs may not synthesise or secrete chitinase, their intestinal microbes do produce chitinolytic enzymes to some extent.

Table 2:12 further indicates that the calcium intake for the pigs fed the larvae meal diet was significantly greater (P<0.06) than those fed on the soybean meal diet. However, even though the faecal and urinary excretion, digestibility and balance values were larger in absolute terms, there were no significant comparative differences (P>0.06) in these factors when expressed as a percentage of intake. This indicates that the calcium contained in the larvae meal was just as available as the calcium contained in the soybean meal. Table 2:12 also indicates that the phosphorus intake, digestibility and balance was significantly greater (P<0.06) for those pigs fed the soybean meal diet. Even when expressed as a percentage of intake, the faecal excretion of phosphorus was greater (P<0.06) and the absorption and balance smaller for those fed the larvae meal diet (Newton *et al*., 1977). Combs *et al*. (1966) and Newman *et al*. (1967) both reported that increasing the dietary levels of calcium would decrease the absorption of phosphorus, while increasing dietary fat had the opposite effect and in fact increased phosphorus absorption.

2.4.1.2 Palatability and intake of black soldier fly larvae meal

Newton *et al*. (1977) reported from their study that the larvae meal and soybean meal diets were both consumed in similar quantities, where the mean daily intake for the pigs of the diets was 169 g and 175 g respectively. There were no clear signs of discrimination between the two diets, where two pens of pigs showed preference for the soybean meal diet, one pen of pigs showed a definite preference to the larvae meal diet and the remaining pen showed no distinct preference between the two diets. From the data, Newton *et al*. (1977) reported that larvae meal diets are just as palatable as soybean meal diets.

2.5 Blood parameters of pigs

The haematological and biochemical parameters of pigs are influenced by a variety of environmental and physiological factors, which include genetics, diet, age, sex, physiological stage and housing (Tumbleson and Scholl, 1981). Advances in technology has allowed for dramatic changes in the production of pigs, as well as improvements in the laboratory techniques used to accurately quantify blood parameters (Wilson *et al*., 1972). The haematological and biochemical profiling of random animals within a farming system provides a valuable indication of the clinical health of the overall herd (Friendship *et al*., 1984), where parameters are strongly influenced by diseases and nutritional deficiencies (Mills, 1974). In the case of the detection of certain trace element deficiencies, measuring the activity of dependant enzymes provides an indication as to what deficiencies are present (Mills, 1974). This provides as an early warning signal for farmers to alter the diets of the animals to ensure that all the nutritional requirements of the animals, for both maintenance and production, are fully satisfied (Church *et al*., 1984; Maxwell *et al*., 1990; Etim *et al*., 2014).

With these concepts in knowledge, especially the fact that diet has an influence on the blood parameters of any animal and that there have been reports of insect larvae meal having positive

effects on the health of broilers (Pieterse, 2014; Pieterse *et al*., 2014; Uushona, 2015), there is enough evidence to warrant the investigation of the influence of BSFLM on the blood parameters (possible immunological gains) of other animals. Also, as mentioned in the chemical composition part of this chapter, the BSFLM had substantially higher concentrations of calcium and iron when compared to other protein sources; thus also providing a warrant for the investigation of the bioavailability of these minerals associated with the inclusion of BSF larvae in animal diets. Therefore, in the case of this study, the focus was on the possible health benefits and the chemical availability of the minerals correlated with the inclusion of BSFLM in piglet diets.

2.5.1 Blood collection

2.5.1.1 Blood sampling techniques

There are various blood sampling techniques that can be undertaken for blood collection and Farmstad *et al*. (1988) described those that can be performed via the ear vein, tail vessels and jugular vein. The ear vein is considered an easy means of collecting samples from pigs of any size and this technique invloves the ear being lanced and blood collected. However, this only allows for small samples to be collected and the chance of sample contamination could arise (Framstad *et al*., 1988). Haemorrhaging of the ear may also follow, which would require pressure applied to relieve blood loss. The tail vessels method can also be utilized, especially in piglets at time of tail docking, as the blood vessel lies close to the skin surface allowing for easy access with the puncture site at the first freely movable tail joint (Framstad *et al*., 1988). The jugular vein method is probably one of the most commonly used blood collection techniques, as this method can be used for pigs of all ages, but specifically adult pigs (Framstad *et al*., 1988). The pig is restrained and its head raised to define the deepest part of the jugular groove and the vein is found between the medial sternocephalic and lateral brachiocephalic muscles (Framstad *et al*., 1988). The trick is to hold the pig in the correct position and to direct the needle perpendicular to the skin, caudo-dorsally (Framstad *et al*., 1988).

Further, Diehl *et al*. (2001) describes the method of blood collection via the anterior vena cava that can be utilized in pigs of all ages for the collection of large samples. A small pig is restrained on its back with the front legs held back and the chin pressed downwards, as this allows for the right hand inlet of the piglet between the first rib and breast bone to be determined (Diehl *et al*., 2001). Older pigs can be bled in a standing position, where the needle is inserted alongside the front of the breast bone directed slightly inwards towards the spine and upwards at a backward angle (Diehl *et al*., 2001). Care should be taken into not swinging the needle which may cause the tearing of the blood vessel and cause haemorrhage and even death (Diehl *et al*., 2001).

2.5.1.2 Blood volume collection and recovery time

Blood withdrawal volume and the allowed recovery period between frequent blood collections is incredibly important for the health and overall wellbeing of the animal (Diehl *et al*., 2001). The maximum allowable volume able to be collected decreases with a decrease in the amount of recovery time given or the increase in the frequency of withdrawal (McGill and Rowan, 1989). It only takes approximately 24 h for the blood volume of an animal to be restored, however, it can take a lot longer for red blood cells, platelets, coagulation factors and other blood contents to be fully replenished (McGill and Rowan, 1989). If too frequent blood collections are administered, then there is a chance of associated abnormalities, such as anaemia, to occur (McGill and Rowan, 1989).

McGill and Rowan (1989) reported that there are two methods of calculating the maximum allowable blood volume collected, where the first uses body weight and the second uses Circulating Blood Volume (CBV). Table 2:13 provides guidelines based on the sample volume and the frequency of withdrawals with regards to these two methods.

Table 2:13 Maximum allowable blood volume withdrawn as calculated by body weight or CBV, given recovery time (Adapted from McGill and Rowan, 1989).

Recovery time	Body weight (%)	Circulating Blood Volume* (%)
3 weeks	1	15
2 weeks	0.75	10
1 week	0.5	7.5
Daily (24 h)	0.05	0.75

*CBV (Circulating Blood Volume) is approximately 6% or body weight

It is noted that the blood volume calculation will be in litres or millilitres, depending on the units of weight used (kg = L; g = mL). If body weight was utilized for the blood volume calculation, then a piglet weighing 1200 g with a recovery period of 2 weeks would allow for a maximum of 9 mL (0.75% x 1200 g) to be withdrawn. However, if CBV was utilized for the blood volume calculation, then a piglet weighing 1200 g with 72 mL (6% x 1200 g) of CBV and a recovery period of 2 weeks, would allow for a maximum of 7.2 mL (10% x 72 mL = 7.2 mL) to be withdrawn.

2.5.2 Brief overview of blood components

2.5.2.1 Haematological parameters

White blood cells (WBCs)

White blood cells or leukocytes are the cells of the immune system that are involved in protecting the body against infectious diseases and foreign invaders and they are produced in the bone marrow and derived from a hematopoietic stem cell, a multipotent cell (Maton *et al*., 2008). Leukocytes are found throughout the body of both animals and humans, including the blood and lymphatic system (Maton *et al*., 2008). The number of leukocytes in the blood is often an indicator of disease or a physiological stress of the body and is usually measured as an absolute number per litre (Alberts *et al*., 2002). There are 5 types of leukocytes that exist and they are distinguished according to their physical and functional characteristics and they are usually expressed as a percentage of the total WBC count (Maton *et al*., 2008).

I. **Neutrophils** are the most abundant WBCs and they form an essential part of the innate immune system, where they defend the body against bacterial or fungal infection (Maton *et al*., 2008). They are usually the first responders to inflammation, particularly microbial infection, and are active in phagocytising bacteria, where they die as they are unable to renew lysosomes (enzymes used in digesting microbes) forming pus (Saladin *et al*., 2012).
II. **Lymphocytes** are more common in the lymphatic system than in blood and they include natural killer cells (function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity) (Saladin *et al*., 2012).

III. **Monocytes** are the largest type of WBC and share the phagocytosis function of Neutrophils, where they also form part of the innate immune system (Maton *et al*., 2008). However unlike Neutrophils, they present pieces of pathogens to T cells so that the pathogens may be recognized and killed (Saladin *et al*., 2012). They eventually leave the bloodstream and become tissue macrophages, which remove dead cell debris and attack microorganisms (Saladin *et al*., 2012).

IV. **Eosinophils** are rare in the blood, but numerous in the mucous membranes of the respiratory, digestive, and lower urinary tracts (Maton *et al*., 2008). Their numbers fluctuate between days and seasons and during menstruation, where they arise in response to allergies, parasitic infections, collagen diseases, and disease of the spleen and central nervous system (Saladin *et al*., 2012).

V. **Basophils** are mostly responsible for allergic and antigen response by releasing the chemicals histamine and heparin (Maton *et al*., 2008). Histamine is responsible for widening the blood vessels and increasing the flow of blood to injured tissue and it also makes blood vessels more permeable to neutrophils and clotting proteins, so that they can get into connective tissue more easily (Saladin *et al*., 2012). Heparin is an anticoagulant that inhibits blood clotting and promotes the movement of white blood cells into a site of infection (Saladin *et al*., 2012). They are the rarest of WBCs and share physicochemical properties with other blood cells making them difficult to study (Maton *et al*., 2008).

Red blood cells (RBCs)

Red blood cells, or erythrocytes, are the most common type of blood cell and their principal function is the delivery of oxygen (O_2) to the body tissues via blood flow through the circulatory system (Maton *et al*., 2008). They lack a cell nucleus and most organelles in order to provide maximum space for the accommodation of haemoglobin and are usually measured as an absolute number per litre (Maton *et al*., 2008).

Haemoglobin (HGB)

Haemoglobin is the iron-containing oxygen-transport metalloprotein in RBCs of all vertebrates and is responsible for the red colour of the RBC and usually measured in grams per decilitre (g/dL) (Maton *et al*., 2008). In the blood, it carries oxygen from the respiratory organs (lungs or gills) to the tissues of the body (Maton *et al*., 2008). The mammalian haemoglobin molecule can bind or carry up to four oxygen molecules at a time. Haemoglobin is also involved in the transport of other gases where it carries some of the body's respiratory carbon dioxide (approximately 10% of the total) as carbaminohemoglobin, in which CO_2 is bound to the globin protein (Saladin *et al*., 2012). It also carries the nitric oxide (important regulatory molecule) bound to a globin protein thiol group, where it releases it at the same time as oxygen (Saladin *et al*., 2012).

Haematocrit (HCT)

Haematocrit or Packed Cell Volume (PCV) is the volume percentage of RBCs in the blood and is expressed as a percentage (Maton *et al*., 2008). As the sole purpose of the RBC is to transfer oxygen from the lungs to the tissues, the blood sample's haematocrit percentage is the point of reference of its efficiency in the delivery of oxygen (Maton *et al*., 2008).

Mean corpuscular volume (MCV)

The mean corpuscular volume, or mean cell volume, is a measure of the average volume of RBCs per volume blood and is measured in femtolitres (fL) (Maton *et al*., 2008). It is calculated using Equation 2:1.

Equation 2:1

$$\text{MCV} = \frac{\text{Haematocrit \%}}{\text{RBC per Volume}}$$

Mean corpuscular haemoglobin (MCH)

The mean corpuscular haemoglobin, or mean cell haemoglobin, is the average mass of haemoglobin per red blood cell in a sample of blood and is measured on picograms (pg) (Maton *et al*., 2008). It is calculated using Equation 2:2.

Equation 2:2

$$\text{MCH} = \frac{\text{Haemoglobin} \times 10}{\text{RBC}}$$

Mean corpuscular haemoglobin concentration (MCHC)

Mean corpuscular haemoglobin concentration is a measure of the concentration of haemoglobin in a given volume of packed red blood cells and is usually measure in grams per decilitre (g/dL) (Maton *et al*., 2008). It is calculated using Equation 2:3.

Equation 2:3

$$\text{MCHC} = \frac{\text{Haemoglobin}}{\text{Haematocrit}}$$

Red blood cell distribution width (RDW)

Red blood cell distribution width is a measure of the range of variation of in the red blood cell (RBC) volume and is expressed as a percentage (Maton *et al*., 2008). Certain disorders cause a significant variation in cell size, where higher RDW values indicate a greater variation in size (Saladin *et al*., 2012).

Platelets

Platelets, or thrombocytes, are a component of blood whose function (along with the coagulation factors) is to contribute to haemostasis, which is the process of stopping bleeding by clumping together and clogging blood vessel injuries (Maton *et al*., 2008). The formation of this platelet plug (primary haemostasis) is associated with the activation of the coagulation cascade, resulting in the fibrin deposition and linking (secondary haemostasis) at the site of injury (Saladin *et al*., 2012). Platelets are the smallest blood cells and have no cell nucleus and are fragments of cytoplasm, which are derived from the megakaryocytes of the bone marrow, and then enter the circulatory system (Saladin *et al*., 2012). They are measured as an absolute number per litre.

Mean platelet volume (MPV)

Mean platelet volume is a measurement of the average size of platelets found in blood and is measured as femtolitres (fL) (Maton *et al*., 2008). A higher MPV provides a good indication of better platelet function, however, there are some medical conditions that are associated with a high MPV and some are associated with a low MPV (Saladin *et al*., 2012). Thus, the MPV value can sometimes be helpful in distinguishing between the different disorders.

2.5.2.2 Biochemical parameters

There are many parameters that can be tested with regards to the biochemical composition of the blood, which includes that of minerals and other metabolites. Thus for this review, only those that were tested in the current study will be described and reasons for testing of these specific parameters are discussed in the respective chapter (Chapter 5).

Minerals

The mineral content in the blood can be tested to achieve an indication as to what extent the body is utilizing the minerals provided in the diet or to what extent these minerals are readily available for absorption for their use in bodily functions (bioavailability) (Bangert *et al*., 2008). This along with digestibility tests serves as a good indication as to the nutritional status of the diet provided for and utilized by the animal. Usually measured in grams per litre (g/L) or millimoles per litre (mmol/L).

Immunoglobulins

Immunoglobulins, or antibodies, are important glycoprotein molecules produced by white blood cells and they play a crucial role in the humoral immune response by binding to bacteria or viruses and aiding in their destruction (Litman *et al*., 1993). They are measured in grams per litre (g/L) and there are five different isotypes.

I. **IgA** is found in the mucosal areas, such as the gut, respiratory tract and urogenital tract and also in saliva, tears and breast milk. The primary role of IgA is to prevent the colonization of pathogens (Litman *et al*., 1993).
II. **IgD** serves mainly as an antigen receptor on B cells that have not been exposed to antigens. It aids in the activation of basophils and mast cells to produce antimicrobial factors (Litman *et al*., 1993).
III. **IgE** binds to allergens and triggers histamine release from basophils and mast cells in response to allergies and also protects against parasitic worms (Litman *et al*., 1993).
IV. **IgG** provides the majority of antibody-based immunity against foreign pathogens and it is the only antibody capable of crossing the placenta to give passive immunity to the foetus (Litman *et al*., 1993).
V. **IgM** is expressed on the surface of B cells (monomer) and in the secreted form (pentamer) with very high avidity. It eliminates pathogens in the early stages of B cell-mediated (humoral) immunity before there is sufficient IgG (Litman *et al*., 1993).

2.5.2 Physiological relationship between nutrition and blood parameters

The nutritional level (implies both quantity and quality of the diet and its components), along with the influence of other factors, is one of the most important elements which may affect the physiology of animals, including blood parameters (Ajao *et al*., 2013). Diets have been reported to have certain measurable effects on blood parameters and the latter can be utilized in the nutritional evaluation of both the dietary components and the accompanied performance of the herd animals (Church *et al*., 1984). Blood haematology and biochemistry analyses allows for a fast means of assessing the clinical and nutritional health status of animals in feeding trials, as well as allows for a fast and necessary adjustment of diets applied within a farming system when animals are not performing optimally (Maxwell *et al*., 1990). Oke *et al*. (2007) stated that the blood transports nutrients to the different parts of the body, therefore whatever effects the blood parameters will certainly have an effect on the entire function of the body in terms of health, growth, maintenance and reproduction. The haematological components, which consists of red blood cells (RBC) or erythrocytes, white blood cells (WBC) or leukocytes, mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), are valuable in monitoring feed toxicity, especially with regards to the feed components that affect the blood as well as the health status of the production animals (Isaac *et al*., 2013). Further, Etim *et al*. (2014) reported that, along with these parameters, packed cell volume (PCV) or haemoglobin may also be correlated with the nutritional status of the animal.

Aslan *et al*. (2002) reported that deficiencies of Vitamin B_{12} or folate produce a macrocytic anaemia in which the red blood cell distribution width (RDW) value is elevated in approximately two out of three of all cases. However, when there is a varied size distribution of RBCs, it is usually associated with iron deficiency anaemia, and as such shows an increased RDW value in almost all cases. In the case of both an iron and B_{12} deficiency, there will normally be a mixture of both large and small cells, also causing the RDW to be elevated (Aslan *et al*., 2002). This elevation in the RDW value caused by red blood cells of unequal sizes is known as anisocytosis.

Furthermore, the blood biochemical parameters offer as a good indication as to the bioavailability of minerals, such as iron and calcium, as well as to what effect diet ingredients have on the physiological status of the animal (Schiavon *et al*., 2000; Bangert *et al*., 2008).

2.5.3 The effect of different diets on the blood parameters of pigs

There is a significant difference between feeding for nutritional requirements and feeding for the relief of hunger (feeling of a full stomach). The difference between these two scenarios is correlated with the nutritional density of the diet, where the animal could experience either a full stomach without acquiring sufficient nutrients to provide for its requirements or could acquire nutrients to satisfy its needs, but remain hungry. It is therefore incredibly important to avoid both scenarios and fulfil the combination of the two, as to provide for nutritional requirements as well as the relief of hunger. Furthermore, physiological variation can also be associated with the different ingredients between diets or the different inclusion levels of the same ingredients between diets, even if the requirements of the animal are met and held constant between the diets. This may be correlated with the varying bioavailability of nutrients of different ingredients and diets (Reece *et al*., 1995).

There have been various studies conducted to evaluate the effect of nutrition on blood haematological parameters. Ani *et al*. (2013) reported on the response of weaner pigs to diets containing 0, 10, 15 and 20% soybean hull, where blood samples were collected for haematological

evaluation and results showed that the diets had no adverse effects on the blood parameters of the pigs. However, in a trial by Fasuyi and Ibitayo (2010), pigs were fed varying levels of wild sunflower leaf meal based diets with inclusion levels of 0, 10, 20 and 30% and it was discovered that there were also no significant differences in the packed cell volume (PCV), neutrophil and monocyte concentrations, but there were significant differences seen in the counts of white blood cells (WBCs), lymphocytes and eosinophils. It was also concluded that pigs could survive and perform well without any toxicological implications within an inclusion level not exceeding 20% of the leaf meal as a protein supplement in commercial pig production.

There have also been various studies conducted to evaluate the effect of nutrition on blood biochemical parameters. Yue and Qiao (2008) conducted a study to determine and compare the effects of a low protein diet (18.6%), supplemented with crystalline amino acids, to a control diet (21.1%) on the biochemical parameters of piglets from weaning to 6 weeks of age. They discovered that the decrease in the dietary crude protein content caused a significant decrease in serum urea, thus, indicating an increase in the biological value of the respective feed, as a lower blood urea nitrogen is a sign of a higher availability of dietary nitrogen (Figueroa *et al*., 2002). Schiavon *et al*. (2000) also performed a trial which resulted in a change in blood biochemical concentrations, where a pre-starter and starter diet were supplemented with trace elements either given as sulphates or proteinates at a common level or a reduced level of inclusion. The common level supplied 278, 148, 315 and 98 mg/kg and the reduced level supplied 128, 38, 135 and 50 mg/kg of iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn), respectively. The results showed that the proteinates significantly increased blood plasma levels of iron, copper and zinc when compared with the sulphates.

These studies show that significant differences in the blood parameters can be experienced by providing production pigs with diets of differing ingredients, as well as diets with varying inclusion levels of the same feed ingredients. Trials performed with varying inclusion levels of a specific ingredient offers as an evaluation of the possible associated effects on blood haematological and biochemical levels, and for the opportunity to discover its optimal inclusion rate.

2.5.4 Black soldier fly larvae and blood parameters

No research has been conducted on the effect of *H.illucens* on pig blood parameters and in fact very little, if any, literature is available in the use thereof in other animal species' diets. Thus, for this literature review, only normal haematological and biochemical reference intervals will be discussed further. However, only limited information has been published on normal porcine blood reference values, as its value as a technique for the evaluation of animal herd clinical health and nutritional status has not yet been fully explored (Friendship *et al*., 1984; Coronado, 2014).

2.5.5 Haematological values for pigs and their significance

As discussed previously, the physiological and nutritional status of animals could cause differences in blood parameters, especially in the values observed for PCV and mean corpuscular volume (MCV) (Etim *et al*., 2014). Togun *et al*., (2007) reported that an increase in PCV along with the marginal increase in RBC is an indication of more efficient erythropoiesis[4] in the experimental animals. In regards to the latter, Nwanbe and Elechi (2009) reported that higher values for PCV and

[4] Erythropoiesis is the production of red blood cells.

haemoglobin (HGB) implies that there is a high level of blood dilution coupled with a low efficiency of oxygen transportation. If the haematological values fall within the normal ranges reported for that animal, it is an indication that the diets did not have any significant influence on the haematological parameters during the experimental period (Togun *et al*., 2007). However, if values fall below the normal ranges, then it could be seen as an indication of anaemia. Bawala *et al*. (2007) reported that low values for haematological parameters could be related to the harmful effects of high dietary contents, contradictory to the latter, Copland (1976) stated that the possible cause of lower values in pigs could be correlated with malnutrition.

The immunological status of an animal is a function of leukocytes (WBC), neutrophils and lymphocytes, where lymphocytes are known to play a primary role in the immune system of both man and animals (Ameen *et al*., 2007). According to Copland (1976), a significantly higher leukocyte count for a pig is thought to be associated with chronic pneumonia or parasitism. If the leukocyte, neutrophil and lymphocyte counts fall within their normal ranges, then it is an indication that the nutritional source did not affect the immune system. If accounted for during early signs, most immunological abnormalities observed in malnutritioned animals can usually be corrected after a period of rehabilitation (Ameen *et al*., 2007). An increase in the neutrophils: lymphocytes ratio could be seen as a good indicator of stress (Minka and Ayo, 2007), which could in fact be a nutritional stress on the animal (Etim *et al*., 2014). However, Eheba *et al*. (2008) reported that a decrease in the WBC count could reflect a possible fall in the production of the defensive mechanism to combat infection. Togun *et al*. (2007) and Campbell and Lasley (1975) reported further, that a significantly lower lymphocyte count could be an indication of a reduction in the ability of animals to produce and release immunoglobulins to battle infections when they occur.

2.5.6 Haematological and biochemical reference ranges in pigs

Table 2:14 illustrates the variance in the haematology reference ranges between different authors for pigs. These differences are associated with the many factors that have an effect on the blood parameters of the animal, which include stress (Minka and Ayo, 2007), diet (Etim *et al*., 2014), season (Chmielowiec-Korzeniowska *et al*., 2012) and physiological factors (Tumbleson and Scholl, 1981).

Table 2:14 Haematology ranges for pigs of different ages.

Parameter	Unit	Merck Veterinary Manual	Friendship *et al*., 1984	Coronado, 2014
Age		Piglet; did not state age	Pig; did not state age	Pig; did not state age
White Blood Cells	10e9/L	11 - 22	8.7 - 37.9	7 - 22
Neutrophils	%	20 - 70	16.6 - 73.1	28 - 51
Lymphocytes	%	35 - 75	12.5 - 70.1	39 - 62
Monocytes	%	0 - 10	0.0 - 17.0	2 - 10
Eosinophils	%	0 - 15	0.0 - 6.0	5 - 11
Basophils	%	0 - 3	0.0 - 2.0	0 - 2
Red Blood Cells	10e12/L	5 - 7	5.3 - 8.0	5 - 8
Haemoglobin	g/dL	10 - 16	9 - 14	10 - 16
Haematocrit	%	32 - 50	26 - 41	-
Mean Corpuscular Volume	fL	52 - 62	42 - 62	50 - 68
Mean Corpuscular Haemoglobin	pg	17 - 24	14 - 21	17 - 21
Mean Corpuscular Haemoglobin Concentration	g/dL	29 - 34	32 - 36	30 - 34
Red Blood cell Distribution Width	%	-	-	-
Platelets	10e9/L	200 - 500	-	325 - 715
Mean Platelet Volume	fL	-	-	5 - 20

It is noted that the usefulness of reference values published in literature are restricted by the numerous biological (age, stage of production, gender, genetics) and external (stress, season, feed, farming system, handling, vaccinations) variations between animals and by the sampling techniques and analytical differences between laboratories (Friendship *et al*., 1984). As such, the reference ranges are not strict boundaries and should only be used as guidelines (Etim *et al*., 2014). Herd haematological and biochemical profiles have only been rarely utilized in the pig veterinary practise and so for this reason, future studies involving problem herds are warranted in order to assess the value of such a diagnostic technique, however it may come at a high financial expense (Friendship *et al*., 1984). Table 2:15 illustrates the variance in the blood biochemical reference ranges between different authors.

Table 2:15 Biochemical reference ranges for pigs of different ages.

	Unit	Merck Veterinary Manual	Friendship *et al*., 1984	Chmielowiec-Korzeniowska *et al*., 2012	Sigma-Aldrich
Age		Pig; did not state age	Pig; did not state age	Growing pigs; 25 - 110kg	Pig; did not state age
Albumin	g/L	19 - 39	19 - 39	-	-
Calcium	mmol/L	1.78 - 2.9	2.02 - 3.21	-	-
Phosphorus	mmol/L	1.71 - 3.1	1.46 - 3.45	-	-
Iron	mmol/L	-	3 - 38	-	-
IgG	g/L	-	-	6.40 - 17.09	17 - 24
IgA	g/L	-	-	0.22 - 0.94	1.3 - 1.6
IgM	g/L	-	-	0.84 - 2.41	1.0 - 3.4

Ig= immunoglobulin

2.6 Manure microbiology in pigs

As mentioned, the problem of waste management is a growing concern, especially with the ever increasing large quantities of manure biomass being produced within the agricultural sector (Newton *et al*., 2005). The pathogens associated with these large quantities pose as a potential health risk to both animals and humans if not managed properly (Sobsey *et al*., 1989). Manure and other wastes, such as urine, sloughed feathers, fur, skin and even respiratory secretions, of various agricultural livestock often contain high concentrations (millions to billions per gram) of bacteria and other pathogens (Guselle and Olson, 2001). Agricultural animals such as cattle and pigs, especially in production facilities that harbour hundreds of animals *per capita*, produce incredibly large quantities of concentrated manure that must be effectively managed to minimize the associated environmental and public health risks (Guselle and Olson, 2001). There are numerous methods adopted for the management of manure biomass and pathogen content and BSF larvae may serve as a possible alternative solution to both of these problems (Bondari and Sheppard, 1987; Newton *et al*., 2005).

2.6.1 Pathogens

Animal pathogens pose as a potential risk to human health and include a variety of viruses (such as pig hepatitis E virus), bacteria (such as *Salmonella* spp.), and parasites (such as *Balantidium coli*), some of which are endemic[5] in commercial livestock and difficult to eradicate from both the animals and the facilities in which they are produced (Sobsey *et al*., 1989). Thus, pathogens in manure and other waste products can pose as a potential threat to human and animal health both on and off animal production facilities if the wastes are not adequately treated or contained (Guselle and Olson, 2001). Table 2:16 illustrates the potential pathogen content of animal wastes.

Table 2:16 Some pathogens potentially present in animal wastes (adapted from Sobsey *et al*., 1989).

Virus groups:	Hepatitis E virus (pig), Reoviruses, Rotaviruses, Adenoviruses*, Caliciviruses*, Influenza viruses (Orthomyxoviruses)*
Bacterium groups:	*Salmonella* spp., *Campylobacter* spp., *Escherichia coli* **, *Aeromonas hydrophila***, *Yersinia enterocolitica, Vibrio* spp., *Leptospira* spp., *Listeria* spp., *Staphylococcus* spp.
Parasites (Protozoans):	*Cryptosporidium parvum, Giardia lamblia, Balantidium coli*

*Humans and animals (including pigs) usually have distinct strains of these viruses, but not always.

**Some strains of these bacteria are non-pathogenic and others are pathogenic. The extent to which pathogenic strains occur in animal wastes varies with the animal species and other factors.

In the case of the current study, only the bacterium groups of *Escherichia coli*, *Staphylococcus* spp., *Salmonella* spp. and *Listeria* spp. will be discussed further.

2.6.1.1 *Escherichia coli (E. coli)*

Escherichia coli is a gram-negative, anaerobic, rod-shaped bacterium that is commonly found in warm blooded organisms (Riemann and Cliver, 1998). *E.coli* are present in the faecal matter of humans, wildlife and domestic livestock, although it may also be found in water and soil with only a small proportion (<1%) of the strains of this bacterial group being pathogenic (Riemann and Cliver, 1998). Most strains of *E. coli* that inhabit the intestines of clinically healthy humans and animals are

[5] Endemic implies a pathogen that is present in relatively low levels in the population all the time.

harmless and are in fact a beneficial component of the natural intestinal flora that can benefit their hosts by producing vitamin K_2, and preventing the colonization of the intestine with pathogenic bacteria (Riemann and Cliver, 1998).

There are several different pathogenic strains of *E. coli* and the one that is of zoonotic concern is *E. coli O157:H7*, which produces toxins that can cause serious human illness. All *E. coli* are classified on the basis of the presence or absence of surface antigens (O, H, K) and the numerical system that's helps to distinguish between harmless and harmful bacteria (Riemann and Cliver, 1998). Riemann and Cliver (1998) reported that pathogenic *E. coli*, such as *E. coli O157:H7*, are also classified according to their ability to produce harmful toxins and attach and invade host intestinal epithelial cells. Faecal oral transmission is the major route in which pathogenic strains of the bacterium cause disease. Colibacillosis is a broad term that refers to any infection or disease caused by *E. coli* that can be detected in livestock by severe diarrhea, lameness, stunted growth, inactivity, lack of appetite and water consumption, and unresponsiveness (Barnes *et al*., 2008). *Escherichia coli* is known to commonly cause diahrrea in piglets within a few days after birth until well after weaning and should be managed properly to prevent the chance of Colibacillosis (Barnes *et al*., 2008).

The shedding load of *E. coli* in animals has been associated with season (Van Donkersgoed *et al*., 2001), age and diet (Van Donkersgoed *et al*., 1999), geographical location (Schurman *et al*., 2000) and stocking density and management conditions (Wilson *et al*., 1992). Read *et al*. (1990) reported that the prevalence of *E. coli O157:H7* in healthy pigs has been between 0.4 and 7.5% and that pigs rarely excrete this verotoxin producing strain. *E. coli* may cause a drop in the growth performance in livestock and even cause diarrhoea, especially when animals face challenges such as weaning, viral infections and/or changes in diet (Nagy and Fekete, 1999).

2.6.1.2 *Staphylococcus* spp. *(Staph.)*

Staphylococcus spp is a genus of gram-positive bacteria and includes up to 40 different species where most are harmless and normally reside on the skin and mucous membranes of humans and other animals and they also form a small part of soil microflora (Casey *et al*., 2013). There are two major divisions of the genus *Staphylococcus* which are serarated by whether or not they are able to produce coagulase, an enzyme that can clot blood (Kloos, 1980). Many human bacterial infections are caused by coagulase-positive *Staphylococcus aureus* strains and coagulase-negative strains, such as *Staphylococcus epidermidis*, are those that produce a slime that is able to interfere with immune defences (Kloos, 1980).

Staphylococcus can cause a wide variety of diseases in humans and animals through either toxin production or penetration, where the toxins are a common cause of food poisoning (Kloos, 1980). This bacterium is found in most, if not in all, production systems in which animals are held and people that live near farms or agricultural fields fertilized with manure are also able to become infected with methicillin-resistant *Staphylococcus aureus (MRSA)* bacteria if the correct protective gear is not utilized (Casey *et al*., 2013).

2.6.1.3 *Salmonella* spp.

Salmonella is a genus of rod-shaped bacteria and consists of two species, namely *Salmonella bongori* and *Salmonella enterica* (Tauxe, 1997). It is found in both cold-blooded and warm-blooded animals and in the environment, where certain strains may cause illnesses such as typhoid fever,

paratyphoid fever, and food poisoning (Salmonellosis) (Tauxe, 1997). However, the strains of *S. choleraeusuis*, *S. dublin* and *S. enteritidis* reside in hosts in which they are best adapted and most commonly found in pigs, cattle and poultry respectively (Ekperigin and Nagaraja, 1998).

Salmonella gains entry into the host animal through direct contact with faeces or indirectly through contaminated food. The most common route of entrance into the host is through the host's oral cavity, however, minute pores in the shells of newly laid eggs provides a means of entry into poultry (Ekperigin and Nagaraja, 1998). Once entry is gained, the invading Salmonella is either destroyed by the host's defences or it succeeds in overcoming the host's defences and settles within the host. If infection does not progress into salmonellosis, the Salmonella bacterium may remain in the gastrointestinal tract of the host and become part of the animal's commensal microflora and may be shed in the manure, which may lead to the contamination of the environment and becomes as a source of infection for other animals (Ekperigin and Nagaraja, 1998).

Salmonellosis in domestic livestock has caused an acknowledgeable increase in the occurrence of salmonellosis in humans (Tauxe, 1997), where manure of infected animals have also been known to contaminate feedstuff, water, milk, meat and other plant and animal products (Ahl and Buntain, 1997). The incidence of Salmonella isolated from pig manure is known to range from 8 to 25% (Letellier *et al*., 1999).

2.6.1.4 *Listeria monocytogens*

Listeria monocytogenes form part of the normal intestinal microflora of the distal portion of the gastrointestinal tract of various animal species, including domestic livestock such as cattle, pigs and chickens (Cooper and Walker, 1998). The incidence of *L. monocytogenes* in pigs ranging from 2 to 6% (Weber *et al*., 1995). Listeriosis usually occurs in sporadic cases, however the outbreaks in domestic livestock have been associated with silage inclusion in the animals' diets (Cooper and Walker, 1998). In humans, the outbreak of listeriosis has been associated with contaminated cheeses and processed meats (Cooper and Walker, 1998).

2.6.2 Pathogen reductions by manure management and treatment

The reductions of some pathogens by some animal waste treatment processes have been determined by laboratory analyses and in general the thermal processes of pasteurization, thermophilic digestion and composting are capable of drastically decreasing the pathogen activity (Sobsey *et al*., 1989). However, further studies into the inactivation of pathogens by thermophilic means of manure treatment are recommended to discover the optimum conditions to achieve extensive pathogen reduction (Sobsey *et al*., 1989).

Drying of animal manures is one of the most common management practises adopted by intensive livestock farmers, however, there is little known on the extent of the inactivation of pathogens in the application of this particular method (Farzan *et al*., 2010). The drying of manure to moisture levels of less than 10% has been reported to significantly reduce the activity of pathogens in bio-solids and soils (Sobsey *et al*., 1989). Another method is that of Mesophilic biological treatment processes, but these practises are not likely to reduce pathogen levels unless there are several treatment reactors utilized in series. Therefore, treated manures, effluents and bio-solids from these processes may still contain high concentrations of pathogens (Sobsey *et al*., 1989). Both of these methods requiring thorough research to be conducted as to determine the rate and extent of pathogen inactivation and the optimal means to achieve extensive reduction in the pathogen activity (Sobsey *et al*., 1989).

Chemical treatment of animal manures are done typically by the treatment with lime or other alkaline sources (Guselle and Olson, 2001). This particular method being largely adopted by municipalities for the treatment at sanitation facilities and less so by livestock producers for the treatment of manure (Sobsey *et al*., 1989). Alkaline stabilization for pathogen inactivation has been reported to be highly effective in municipal bio-solids and results that have been achieved in its application in animal manure management show great potential (Sobsey *et al*., 1989).

2.6.3 Black soldier fly larvae and manure management

Biomass management

As previously mentioned, manure can serve as a potential source of nutrients for BSF larvae that have the ability to efficiently convert waste to a valuable protein source (Calvert and Martin, 1969; Newton *et al*., 2005; St-Hilaire *et al*., 2007; Sealey *et al*., 2011; Pieterse *et al*., 2014). Newton *et al*. (2005) reported that black soldier fly larvae reduced 55 kg of fresh manure dry matter to 24 kg of digested manure dry matter within 14 days, which is of a 56% reduction in the waste product. The presence of BSF larvae in manure can also cause a reduction in the moisture content (Calvert and Martin, 1969) and odour release (Teotia and Miller, 1974). Thus, the capability of BSF to reduce the volume and weight of the would-be waste organic matter provides a huge opportunity for its use in effective and eco-friendly waste disposal (Sheppard *et al*., 2002).

Pathogen management

Bondari and Sheppard, (1987) reported that BSF larvae have the potential to significantly reduce the pathogen counts of *E. coli* and *S. enterica* by modifying the micro flora of the manure. Just as animals produce antibodies in response to bacterial and viral infections, lower forms of animals (including *H. illucens*) also possess the ability to chemically defend themselves from microbes, although the molecules may be simpler than mammalian antibodies (Sheppard *et al*., 1994). These may take the form of antibacterial proteins or peptides which are always present in a particular species which most commonly competes with specific microbes for a food source, or requires that a specific microflora be maintained in its digestive tract (Sheppard *et al*., 1994). Also, in addition to these always present molecules, lower animals also have a range of inducible antibacterial peptides which are produced in the presence of attack or infection. These inducible peptides being effective against families of microbes rather than a specific microbial species (Sheppard *et al*., 1994).

Erickson *et al*. (2004) reported that BSF larvae did lead to the deactivation of *E. coli O157:H7* in poultry manure, but did not reduce its activity in bovine and pig manure. Contradictory to these findings, Liu *et al*. (2008) reported that BSF larvae did in fact reduce the activity of *E. coli O157:H7* in dairy cattle manure, however, the ability of the larvae to effectively reduce counts was significantly affected by the larval-manure loading capacity. Additionally, the temperature also had a significant influence on the pathogen reduction ability of the BSF larvae, where maximum reduction was achieved at 27 to 31°C (Liu *et al*., 2008). This phenomenon could be a combination of both temperature and the processing ability of BSF larvae on manure, as temperatures were not an ideal environment for the *E. coli* (Hsiao-Hui *et al*. 2006). Other factors such as manure type, pH and moisture content also have significant effects on pathogen survival and proliferation and the correlation with BSF manure pathogen reduction (Erickson *et al*. 2004).

2.7 Cost effectiveness/ feasibility of black soldier fly larvae

The production of larvae meal has the potential to be a cost effective, alternative source of protein when compared to those already existing sources in the market. Newton *et al*. (2005) compared the cost of larvae meal production with fish meal and soybean meal. Pigs are expected to produce approximately 11 kilograms of manure total solids (dry matter) per 1000 kilograms of animal weight per day (ASAE Standard D384.1, 2003). Thus, for a 1000 grow to finish pigs from 22 to 115 kilograms over 110 days, the manure dry matter would be expected to be about 85 tonnes. In various publications, black soldier fly larvae have converted pig manure dry matter to prepupae dry matter at rates ranging from 12 to 16%. If one was to consider only 12% of 85 tonnes of manure to be converted, then it would yield ~11.25 tonnes of dried prepupae (Newton *et al*., 2005). Therefore, if dried prepupae had of similar value to fish meal, then 11.25 tonnes would have a value of R 225,575.33 (R 20,051.14 per ton) and if it had a value equal to soybean meal (R 4,785.47 per ton), then 11.25 tonnes would have a value of R 53,836.54. At a 16% conversion rate, these values would be R 272,695.50 and R 65,082.39, respectively (Prices per ton as taken from IndexMundi.com, accessed on 2 September 2015).

Also, Fashina-Bombata and Balogun (1997) completed a study where they too compared the cost of the larvae meal production with that of fish meal and they reported that the cost of growing, harvesting and processing the larvae meal was less than 20% of the cost of a similar weight in fish meal. Ajani *et al.* (2004) in a later study reported that the replacement of fish meal with 50% and 100% larvae meal has led to a reduction in cost of tilapia production by 18% and 28%, respectively.

2.8 Conclusion

From the literature review it can be concluded that insects belonging to the order Diptera of the Stratiomyidae family show great potential as an alternative protein source that can partially replace traditional protein sources used in animal nutrition. *Hermetia illucens* larvae meal has proved to be a suitable protein source for its inclusion in pig diets, as well as other animal species. It has a high crude protein content that ranges from 30.63 to 43.60% and ether extract content ranging from 26.00 to 44.72%. The larvae meal has a good amino acid profile when compared to that of fish meal, soybean oilcake meal and sunflower oilcake meal. There were differences observed between larvae meal and soybean meal in the research by Newton *et al*. (2004), however, the shortcomings could be overcome by feeding larvae meal in combination with other protein sources. The addition of crystalline amino acids to pig diets may also help achieve the ideal amino acid profile required for optimal production. The use of insects as an alternative protein source in animal diets is humane and reported to have no significant effects ($P>0.05$) on the performance of the animals and to be especially valuable in terms of amino acid, ether extract, calcium and iron content.

Consumer perception with regards to the use of non-traditional feed ingredients as animal feeds might be a challenge, especially with the worldwide growing concern on animal welfare. Black soldier fly larvae has been reported to be a useful method in the processing of waste biomass, as it has the potential to decrease the quantity of the medium (manure) in which pathogens take refuge. It has also being correlated with a decrease in the actual count of certain bacteria within the manure. Black soldier fly larvae accelerate the reduction of *E.coli* and *Salmonella spp.* in the manure, which would suggest that BSF treatment removes zoonotic *Enterobacteriaceae*. Thus, decreasing the risk of

disease transmission to animals and humans when the manure by-product is used as fertiliser, which is no longer ‘too rich’ in nutrients to apply to crops.

The impacts, from both an environmental and economic perspective, of a widespread adoption of this nutrient recirculation system could prove extremely beneficial. The value of the larvae or prepupae meal produced should provide a strong incentive to undertake and manage this system well according to the report by Sheppard and Newton (1994). The potential added value from converting agricultural waste to a high value feedstuff could turnover millions of Rands in South Africa alone, and if undertaken by the rest of the world could have positive consequent impacts on the environment, countries’ economies in terms of waste management and the animal feed market. If the larvae and prepupae meal were to be marketed as speciality feeds, or processed further for the production of biofuel or other possible products, then the value could be much higher. However, there are likely to be restrictions on the usage and trade of products derived from larvae fed animal waste.

Thus, the nutrient recirculation system offers as a progressive and beneficial program to undertake, but requires further research to be conducted to determine the full potential of its use in the world of today. If BSFLM proves to be a viable protein source in piglet diets, this in turn would increase the availability of protein sources for animal production and reduce competition with commodities that could otherwise be used for human consumption. Black soldier fly larvae may prove to be a method of achieving a sustainable farming system with the benefit of increasing meat protein available for the increasing human population.

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Chapter 3

Proximate analysis and amino acid composition of black soldier fly (*Hermetia illucens)* larvae meal and the effect of its inclusion on the production parameters of piglets.

3.1 Abstract

The effect of black soldier fly (*Hermetia illucens*) larvae meal supplementation on nursery pig performance of three hundred and fifteen piglets was investigated utilizing a block design consisting of two treatments (a control diet with 0% larvae meal inclusion and an inclusion diet with 3.5% larvae meal of the total volume). Where the proximate analysis of *H. illucens* showed that it contained, on a dry matter basis, a crude protein content of 35.9%, 48.1% crude fat, 6.5% crude fibre and 7.8% ash. *H. illucens* larvae meal supplementation in a four week phase-over feeding scheme achieved results that showed no difference (P>0.05) between the inclusion diet when compared to the control. As litter average piglet live weights, cumulative feed intakes and average daily gains at weaning (28 days of age) were 6.579 kg and 6.737 kg, 0.282 kg and 0.276 kg and, 0.199 kg and 0.203 kg for the inclusion and control diets, respectively. The results also showed that similar performance (P>0.05) characteristics were achieved between the two treatment diets when the litter average piglet (litter total divided by number of piglets) and the selected piglet (selected individual piglet that fell closest to the calculated litter average) were compared. Thus, it can be concluded that BSFLM can be successfully utilized as an alternative protein source to partially replace protein sources as pertaining to maintaining piglet performance.

Keywords: pigs, piglets, production, larvae meal, BSF, ADG, feed intake

3.2 Introduction

Protein is an extremely important ingredient required in animal nutrition. Over the years there has been great emphasis placed on the quality of proteins included in the diets of pigs, especially the amino acid composition of the variable protein sources (Ellinger, 1958; Reece *et al*., 1995; PIC, 2008). Livestock farmers are selective in protein sources according to the quality, but also according to the cost that is associated with the purchase of a particular protein source. Due to the lack of renewable protein sources along with the rise in protein feed costs, it is becoming increasingly important to find new good quality alternative and sustainable protein sources (Téguia *et al*., 2002; Newton *et al*, 2005). Therefore, there is much needed research to be conducted into alternative protein sources that are both renewable and affordable, so that farmers can both minimize their costs of production, while maximizing their profits. *Hermetia illucens* can be considered as such a protein source. In the few studies that have been conducted, reports on the evaluation of fly larvae meal have led it to be considered to be a complete or partial replacement of other protein sources in animal diets. These include the well-known protein ingredients of fish meal (Téguia *et al*., 2002) and soybean oil cake meal (Hwangbo *et al*., 2009).

The source of protein is a very important factor for growing piglets because feeds with poor amino acid profiles negatively affect their health, development and production (Ellinger, 1958). There have been trials performed on the inclusion of different levels of protein in the diets of pigs (Bindas *et al*., 2009), however, the literature of the latter had little relevance to this specific study, as the protein levels were held constant between the two treatments diets fed (22%). The only difference between the diets in this study was that BSFLM was used to partially replace commercially used protein sources in the inclusion diet. There have not yet been any reports on the inclusion of BSFLM in piglet diets, thus, the literature available is very limiting and the only studies of interest were those reported by Newton *et al.* (1977) and Newton *et al*. (2005). In the study by Newton *et al.* (1977), the digestibility and palatability of the BSFLM was evaluated and it was discovered to have lower dry matter, nitrogen, calcium and phosphorus digestibilities when compared to a soybean meal diet. In the palatability trial, the pigs showed no discrimination between the soybean meal and BSFLM diets (Newton *et al.*, 1977). Newton *et al*. (2005) reported that BSF larvae are able to convert manure into a high value feedstuff that can be a potential method of waste management and serve as a nutritional source in animal diets.

Insect meal is prepared from either larvae, pupae or prepupae and has been reported to contain high levels of protein and has proved to have great nutritional potential in humans (Ramos-Elorduy, 1997; van Huis, 2013), poultry (Okah and Onwujiariri, 2012; Pieterse *et al*., 2014) and fish diets (Ogunji *et al*., 2008; Aniebo *et al*., 2009). Several authors have reported that insect meal supplementation has improved the performance of both poultry and fish (Sealey *et al*., 2011; Okah and Onwujiariri, 2012; Pieterse *et al*., 2014). This could be caused by not only the high protein content of the insect meal, but to the sufficient concentration of essential amino acids (Newton *et al*., 2005; Hassan *et al*., 2009; Ijaiya and Eko, 2009; Barroso *et al*., 2014; Pieterse *et al*., 2014) and adequate fatty acid composition (Raksakantong *et al*., 2010; Pieterse and Pretorius, 2014) necessary for proper animal growth and development. The chemical composition of insect meals are, however, largely affected by feed substrate (Newton *et al*., 1977; Pieterse *et al*., 2014), processing techniques (Fasakin *et al*., 2003) and stage at harvest (Calvert and Martin, 1969; Inaoka *et al*., 1999; Newton *et al*., 2005; Aniebo *et al*., 2008). Thus, continuous chemical composition analyses are required to accurately assess the nutritional value of the insect meals. Table 3:1 summarizes the nutritional composition of *H.illucens* prepupae and larvae meal as reported by the various authors.

Table 3:1 Comparison of black soldier fly larvae and prepupae composition (DM basis) receiving different feed substrates.

	FAO, 2015	**Newton *et al*., 2005**		**St-Hilaire *et al*., 2007**	**Pieterse, 2014**
Feed Substrate		Pig manure	Poultry Manure	Pig Manure	Kitchen/Food Waste
Stage at Harvest		Prepapue	Prepupae	Prepupae	Larvae
Dry matter (% as fed)	91.30	-	-	91.60	91.30
Crude protein (%DM)	42.10	43.20	42.10	43.60	30.63
Crude fibre (%DM)	7.00	-	7.00	-	11.11
Ether extract (%DM)	26.00	28.00	34.80	33.10	44.72
Ash (%DM)	20.60	16.60	14.60	15.50	8.60
Gross energy (MJ/kg DM)	22.10	-	-	-	22.10

Thus the literature available (regardless of species), along with the on-going pressure to find sustainable protein sources and the lack of larvae feed trials performed within the pig industry, warrants for further research to be conducted, as the possible impact of BSFLM has not yet been fully investigated. Therefore, the objective of this study was to investigate the effect of the inclusion of *H.illucens* larvae meal on the production parameters of piglets with special reference to average daily gain (ADG) and feed intake (FI). It is noted that since there was significant variation in the nutritional composition of BSF larvae between the various authors (Newton *et al*., 1977; Fasakin *et al*., 2003; Newton *et al*., 2005; St-Hilaire *et al*., 2007; Sealey *et al*., 2011; Finke, 2013; Pieterse *et al*., 2014) it was decided to determine the proximate and amino acid composition of the *H. illucens* larvae meal grown on kitchen waste.

3.3 Materials and methods

It is is noted that the materials and methods for the entirety of this study were approved by the Stellenbosch University Reseach Ethics Committee with the ethical clearance number: SU-ACUM14-00033.

3.3.1 Rearing of the black soldier fly larvae

The black soldier fly for this specific study were produced similar to the methods as adopted by Pieterse *et al*. (2014). The larvae required for this trial were produced using kitchen waste and all were grown in a single run. Fly eggs were collected from the breeding holds and placed into a hatchery set at a temperature of 32°C and a relative humidity of 80%. After 24 h, the larvae were transferred onto the growth medium consisting of kitchen waste, such as food service losses from uneaten and damaged products, losses due to past due-date products and transportation losses. The larvae were then harvested after 36 h and post-harvest treatment included killing and drying. Killing was performed by means of freezing at -20°C for 24 h. Larvae were removed from the freezer and allowed to defrost at room temperature before drying in a ventilated oven for 24 h at 65°C until constant weight. After drying, the larvae were milled through a 3-mm sieve using a Foss Knifetec 1095 (Höganäs, Sweden). The milled samples were stored at -20°C until mixing of treatment diets.

3.3.2 Proximate analysis and amino acid composition of *H.illucens* larvae meal

All the analytical methodologies were performed at the Department of Animal Sciences at Stellenbosch University, besides that for the amino acid determinations which was conducted at the Central Analytical Facility (http://academic.sun.ac.za/saf/). Each sample was tested in duplicate with a 5% error difference allowed between the two subsamples and if results did not fall within this error percentage, tests were repeated until the respectable results were achieved.

3.3.2.1 Dry matter and moisture determination

The dry matter (DM) of the larvae meal was determined according to the Association of Official Analytical Chemists International (2002), Official Method 934.01. Two subsamples weighing 2.00 g each were placed in a crucible for drying for 24 h at 100ºC. Thereafter, the dry subsamples were cooled off to room temperature, weighed and the DM content for each was calculated using Equation 3:1.

Equation 3:1

$$\% \text{ Moisture} = \frac{(A + B) - C}{B} \times \frac{100}{1}$$

$$\% \text{ Dry Matter} = 100 - \% \text{ Moisture}$$

Where:

A = Weight of empty and dry crucible (g)

B = Weight of air dried test sample (g)

C = Weight of crucible and moisture free test sample (g)

3.3.2.2 Ash determination

The subsamples retained from the dry matter analysis were utilized for the determination of ash content, where the method was followed as provided by the Association of Official Analytical Chemists International (2002), Official Method 942.05. The two subsamples were placed in a furnace oven for 6 h at a temperature of 500°C. Thereafter, these subsamples were cooled off to room temperature, weighed and the ash content was calculated using Equation 3:2.

Equation 3:2

$$\% \text{ Ash} = \frac{(D - A)}{B} \times \frac{100}{1}$$

$$\% \text{ Organic Matter} = 100 - \% \text{ Ash}$$

Where:

D = Weight of crucible and ash (g)

3.3.2.3 Crude protein determination

The crude protein content of the larvae meal subsamples was determined by measuring the total nitrogen content according to the method described by the Association of Official Analytical Chemists International (2002), Official Method 4.2.07. This method consists of combusting a sample of known mass in a high temperature chamber in the presence of oxygen at about 900°C, leading to the release of carbon dioxide, water vapour and nitrogen. These gases are passed over special columns, where a column containing a thermal conductivity detector at the end separates the nitrogen from any residual carbon dioxide and water vapour. The remaining nitrogen content is then measured and a value is provided by the machine. It is noted that the instrument was first calibrated by a lab technician utilizing pure material of Alfalfa of known nitrogen concentration.

The two subsamples, weighing 0.10 g each, were placed in foil cups and placed into the LECO FP528 apparatus (LECO Corporation, St. Joseph, USA) for analysis. Thereafter, the nitrogen content value provided by the machine was taken and the Crude Protein content was calculated using Equation 3:3.

Equation 3:3

$$\text{Crude Protein} = \text{Nitrogen} \times 6.25$$

3.3.2.4 Crude fibre determination

Fibre is very complex and is a combination of four major components, namely cellulose, hemicellulose, lignin and pectin and gums, which are distinctly different in chemical composition. The crude fibre content was determined according to the method described by the Association of Official Analytical Chemists International (2002), Official Method 962.09. This method determines the crude fibre content gravimetrically, after the other components present (protein, starch and other digestible/soluble carbohydrates) have been chemically digested with diluted sulphuric acid and sodium hydroxide (diluted alkali). The value achieved for the crude fibre content of an insect meal is an indication of the chitin content associated with the insect exoskeleton.

It is noted that the subsamples were first defatted with 20 mL Petroleum ether, according to the method provided by the A.O.A.C., (2002), Official Method 7: Ba 6a-05, prior to the procedure. The two subsamples, weighing 1.00 g each, were placed into a glass crucible and thereafter into the Fibertec/Dosifiber extrusion apparatus. The samples were first boiled in the 0.128M sulphuric acid for a period of 30 min, which removes free sugars and starch. The samples were then washed three times with distilled water and then boiled in 0.313M sodium hydroxide for another 30 min, which removes protein and carbohydrates, after which they were washed three times again with distilled water. Thereafter the completion of the procedure, the subsamples were dried at 100°C for 24 h and then placed in the furnace oven for 6 h at 500°C. The crude fibre content was then calculated using Equation 3:4.

Equation 3:4

$$\% \text{ Crude Fibre} = \frac{A - B}{\text{Sample mass (g)}} \times \frac{100}{1}$$

Where:

A = Mass of residue in crucible after drying (g)

B = Mass of residue in crucible after ashing (g)

3.3.2.5 Crude fat determination

The crude fat content was determined by following the method as provided by Association of Official Analytical Chemists International (2002), Official Method 954.02. The crude fat content is determined by acid hydrolysis with HCl flowed by the extraction of hydrolysed lipid materials with mixed ethers, the ether is then evaporated and the residue is heated to constant weight and expressed as a percentage.

Two subsamples of each sample, weighing 2.00 g each, were placed in a soxhlet fat beaker were put into the drying oven over night at a temperature of 100°C. The subsamples then received 2 mL of ethanol to wet the sample, followed by the addition of 10 mL HCl (38%) and boiled 30-40 min in the water bath. The boiled samples were rinse poured into a separating funnel with 10 mL of ethanol and the following steps were performed:

Step 1:

The addition of 25 mL diethyl ether and shaken for 1 mim.

The addition of 25 mL petroleum ether and shaken for 1 min.

Upper portions poured into the respective fat cups.

Step 2:

The addition of 15 mL diethyl ether and shaken for 1 min.

The addition of 25 mL petroleum ether and shaken for 1 min.

Upper portions poured into the respective fat cups

Step 2 repeated

The fat cups were placed on a sand bath between steps for 30 min to allow the ether to evaporate and after the final evaporation, the crude fat content was calculated using Equation 3:5.

Equation 3:5

$$\% \text{ Crude Fat} = \frac{A - B}{\text{Sample mass (g)}} \times \frac{100}{1}$$

Where:

A = Mass of fat cup plus fat (g)

B = Mass of fat cup (g)

3.3.2.6 Amino acid determination

The amino acid profile of the samples was determined as described by Cunico *et al*., (1986). The samples were first prepared through hydrolysis and then the total amino acid content was determined. The process of hydrolysis involved a sample weighing 0.10 g, which was placed into a specialized tube and 6 mL of 6N Hydrochloric acid and 15% Phenol solution were added to the subsamples. The samples were then placed under a vacuum by utilizing a vacuum pump and nitrogen was added under pressure, after which the tubes were sealed with a blue flame Bunsen burner. These samples were then left to hydrolyse for 24 h at 110°C and after the samples were transferred into Eppendorf tubes and refrigerated until amino acid determination.

The samples were sent to the Central Analytical Facility where the samples underwent a precolumn derivatisation of the amino acids and were separated utilizing High Performance Liquid Chromatography. The amino acid content of the samples was then determined utilizing a fluorescence detector.

3.3.3 Animal selection

Sows were selected as to achieve an even distribution of parities and genetic type between the treatment diets within each block, as well as to achieve an even distribution of litters between the treatment diets (Table 3:2). The original sow 18 (5^{th} parity, Large White, 28-May-15) was replaced as she had nine piglets born dead.

Table 3:2 Sow selection.

Identity	Parity	Genetics	Due Date	Sow number
		Block 1		
Control				
TD 175	6	Large White	21-May-15	1
TD 187	6	Large White	21-May-15	2
TD 195	6	Large White	21-May-15	3
TD 137	6	Large White	22-May-15	4
TD 357	5	Large White	21-May-15	5
TD 392	5	Large White	21-May-15	6
TE 078	3	Landrace	21-May-15	7
TE 084	3	Landrace	21-May-15	8
Inclusion				
TD 186	6	Large White	22-May-15	9
TD 180	6	Large White	23-May-15	10
TD 185	6	Large White	23-May-15	11
TD 197	6	Large White	23-May-15	12
TD 400	5	Large White	21-May-15	13
TD 321	5	Large White	22-May-15	14
TE 074	3	Landrace	21-May-15	15
TE 066	3	Landrace	22-May-15	16
		Block 2		
Control				
TD 144	6	Large White	28-May-15	17
TE 068*	3	Large White	28-May-15	18
TD 333	5	Large White	28-May-15	19
TD 553	4	Large White	28-May-15	20
TD 556	4	Large White	28-May-15	21
TE 042	3	Landrace	28-May-15	22
Inclusion				
TD 190	6	Large White	28-May-15	23
TD 402	5	Large White	28-May-15	24
TD 315	5	Large White	29-May-15	25
TD 558	4	Large White	28-May-15	26
TD 562	4	Large White	29-May-15	27
TE 064	3	Landrace	28-May-15	28

*Original sow (5th parity, Large White, 28-May-15) replaced due to nine piglets born dead.

3.3.4 Animals and housing system

The trial was conducted at Tana Piggery, a pig production farm located near Klapmuts, Western Cape, South Africa. Three hundred and fifteen piglets among the 28 litters were utilized from birth to 28 days of age. The piglets were housed in sealed farrowing units and the internal temperature (25-30°C) was controlled by manual vents and thermal lighting was provided as an external heat source to help piglets regulate their body temperature. The farrowing units comprised of 50 farrowing pens per unit, where thirty sows were artificially inseminated each week and these sows remained with their piglets for a period of 4 weeks after birth. This provided for 30 pens to be vacant each week to

allow for efficient cleaning and disinfecting in preparation for the next group, as this provided for the continuous flow of production animals through the system. The sows were moved into the farrowing house three days prior to farrowing to ensure that the litters were born in the farrowing unit and not the gestation house where piglets could have been trampled. Within each pen, a tube feeder and nipple drinker was supplied for the sow and a self-feeding trough and nipple drinker for the associated litter of piglets. Precautionary measures were adapted into the construction of the farrowing pens, as a crush surrounded each sow which prevented her from accidently killing or injuring one of her young. On completion of farrowing (all piglets born), cross-farrowing was administered within a few hours as to achieve as even distribution of piglets between the litters as possible, thus as to achieve optimal production (larger litters dispersed between smaller litters to limit dominancy and possible consequent variation in growth).

The piglets were tagged, teeth cut and weighed individually at birth and allowed to suckle from their mother *ad lib.* After completion of farrowing and cross-farrowing had been conducted, the litter average mass per piglet was calculated and the piglet that was within the closest range of this average was selected for the collection of individual data, as a representation of its respective litter (individual mass gain, blood withdrawal and faecal collection). The piglets were monitored constantly on a daily basis and any mortalities were recorded, but were not subjected to *post mortem* investigation for the current trial.

3.3.5 Experimental diets

The treatment diets are presented in Table 3:3. The diets were formulated so that the piglets were maintained on at least the minimum nutrient requirements (PIC, 2008), as to achieve proper growth and development of the animals in their early stages of life. In doing so, the maximum allowable BSFLM inclusion was 3.5% of the total diet, as this met the requirements of the animal. The initial feed trial concept was to test the treatment diets of a control (0% inclusion) and inclusion levels of 5, 10 and 15% larvae meal, however in the attempt to formulate for the desired diets, only an inclusion of 3.5% was allowed. The diets were supplied to the animals from 10 days of age and the first 2 days were considered as an adaption period of the animals to the feed. The treatment diets were supplied to the piglets four times a day to prevent any hindrance in intake by foul odours that would have otherwised been absorbed from inside the house.

It is seen from Table 3:3 that fish meal and soybean meal were also included as protein sources for diet formulation, where the inclusion of soybean meal was held constant and the fish meal inclusion differing between the two diets with *Hermetia illucens* larvae meal only partially replacing fish meal.

Table 3:3 Ingredient and calculated nutrient composition of the experimental diets.

	Unit	Control	Inclusion
Ingredients			
Whey powder	%	12.96	14.96
Hp300	%	1.07	5.49
ADVIT std pig creep with natuphos	%	0.40	0.40
Hermetia illucens larvae meal	%		3.50
Maize	%	52.12	50.56
Soybean full fat	%	12.00	7.82
Soybean 46	%	8.00	8.00
Fish meal 65	%	11.32	7.39
L-lysine HCl	%	0.12	0.22
DL methionine	%	0.05	0.08
Premix*	%	0.15	0.15
Limestone	%	0.39	0.21
Monocalcium phosphate	%	0.58	0.78
Sodium bicarbonate	%	0.41	0.46
Oil - soya	%	0.42	
Calculated Nutritional value			
Dry matter	%	89.17	89.43
Metabolizable Energy (pig)	MJ/kg	14.25	14.03
Digestible Energy (pig)	MJ/kg	14.95	14.70
Crude protein	%	22.32	22.17
Crude fibre	%	2.27	2.33
Crude fat	%	6.00	6.00
Ash	%	5.04	4.79
Ether extract	MJ/kg	11.37	11.28
Calcium	%	0.85	0.85
Lysine	%	1.46	1.46
Methionine	%	0.49	0.48
Cystine	%	0.35	0.37
Methionine + Cystine	%	0.85	0.85
Carbohydrates	%	0.25	1.26
Lactose	%	15.00	15.00
Threonine	%	0.91	0.89
Tryptophan	%	0.25	0.25
Arginine	%	1.30	1.13
Isoleucine	%	1.01	0.98
Leucine	%	1.95	1.88
Histidine	%	0.57	0.51
Phenylalanine	%	0.91	0.90
Tyrosine	%	0.74	0.76
Phenylalanine + Tyrosine	%	1.65	1.66
Valine	%	1.12	0.99
Glycine	%	1.22	1.27
Glycine + Serine	%	3.01	3.19
Phosphorous	%	0.79	0.85
Available phosphorous	%	0.55	0.55
Sodium	%	0.35	0.35
Chloride	%	0.40	0.40
Potassium	%	0.89	0.96
Linoleic acid	%	2.47	1.96

*Vitamins and minerals included according to the levels provided by the (National Research Council, 1994).

3.3.6 Experimental design and trial procedure

Three hundred and fifteen piglets were among the 28 litters utilized for the trial, where each litter was present with its mother in the farrowing pen. The 28 litters were placed into two blocks according to their respective farrowing dates, where the two treatments were administered with eight replications per treatment with an average of 11 piglets per replicate for block 1 and six replications per treatment with an average of 11 piglets per replicate for block 2. The piglet that was within the closest range of litter average was then selected for the collection of individual data as a representation of its respective litter (selected piglet).

3.3.6.1 Data collection and analysis

The average piglet mass of each litter and the body mass of the selected piglet were determined at birth and at 10 (introduction of creep feed), 19 (middle of feeding scheme) and 27 (end of trial) days of age. Although it would have been desirable to have more body weight points, the stress associated with these weighings and its potential influence on the production of the piglets did not warrant more regular weighings. Feed was supplied *ad libitum* and daily intake was determined. The data gathered was used for the calculation of daily feed intake per litter, the average intake per piglet and average daily gains (ADG). The formulae used are shown in Equation 3:6 and Equation 3:7.

Assumptions

I. Normality between the piglets within a litter.
II. Normality between the litters.

Equation 3:6

$$\text{Average Daily Gain} = \frac{A}{\text{Age (days)}}$$

Where:

A = litter average mass per piglet or selected piglet mass (kg)

Equation 3:7

Feed intake per litter = Feed weighed in - (Feed weighed out + Feed refusal)

Where:

Feed weighed in = weight of feed container at start of day (before feed supplied) (g).

Feed weighed out = weight of feed container at the end of the day (after feed supplied) (g).

Feed refusal = weight of feed that was not consumed between feeding periods (g).

3.3.6.2 Statistical analysis

Due to the fact that a number of variables were experienced when recording the data, an exploratory analyses was done to determine whether or not their influence was sufficient to warrant their inclusion in the final model for analyses.

These exploratory analyses included fitting the full model and combination of variables using the PROC GLM of SAS for Windows Version 9.3 (statistical software). The statistics were done by using

analysis of covariance with least square means (LSM) calculated with Bonferroni *post hoc* test. A probability of $P<0.05$ was used to determine significance. A full model was initially analysed where the main factor of parities was tested with farrowing dates included as blocks. Piglet age (included as weighing periods) and number of piglets per litter were also included as co-variables in the different models used for analysis. Due to parities, blocks and number of piglets per litter not having significant effects, these were excluded from the models. Breed was also excluded due to the very few Landrace animals included in the experiment.

After exclusion of all these variables, the final model included the variables of piglet age (weighing period) and treatment. Repeated measures of ANOVA with Bonferroni *post hoc* test were completed using PROC MIXED. Average daily gain was calculated by means of a linear regression for each litter, and treatment significance was analysed which included the intercept of each equation as a co-variable in order to adjust for starting weight differences. Descriptive statistics and graphs of treatments and age interaction for the average piglet mass and the selected piglet mass are included to provide a graphical representation of the mass gain over time.

3.4 Results and discussion

3.4.1 Proximate analysis

Table 3:4 summarizes the composition of the BSF larvae in terms of results achieved by proximate analysis. These results were compared with those published in literature and it is noted that the literature values for the crude protein content of BSFLM ranged between 30.6% (Pieterse, 2014) and 43.6% (St-Hilaire *et al*., 2007). The crude protein content obtained in this specific study (35.9%) was comparable to that reported by Pieterse (2014), this may be correlated with the fact that the larvae were reared on the same feed substrate (kitchen waste), harvested at the same stage (larvae) and processed by the same method (oven dried at 60°C). The slight difference may be explained by a difference in day of harvest within the larval stage, for example, Aniebo and Owen (2010) reported that there was a decrease in crude protein content from 59.6% to 54.2% to 50.8% in oven dried house fly larvae harvested at day two, three and four respectively. The crude protein content achieved in the current study, when compared to results achieved by Newton *et al*. (2005) (43.2%) and St-Hilaire *et al*. (2007) (43.6%), was significantly lower. These high crude protein values reported by the respective authors could be related to the difference in larval growth medium, stage at harvest and the method of processing. For example in both reported studies, the BSF larvae were grown on pig manure, harvested as prepupae and dried at 70°C and 80°C, respectively. Furthermore, the larvae utilized in the current study were not only raised on kitchen waste, but also not defatted, thus the relative protein to ether extract values were much higher. Newton *et al*. (2005) also determined a high crude protein content (42.1%) for larvae grown on poultry manure, holding all other factors constant. This could be explained by the fact that poultry manure has a high urea concentration that could attribute to the higher nitrogen values in the larvae, hence a high crude protein content (McDonald, 2002). This difference can also be explained by the fact that the prepupae are covered with a chitin layer that consists of nitrogen- hydrogen bonds (Kramer and Koga, 1986). This higher nitrogen content of the prepupae caused an increase in the calculated protein content, due to the method of analyses utilized; Association of Official Analytical Chemists International (2002), Official Method 4.2.07.

Table 3:4 Results obtained for proximate analysis of the black soldier fly larvae meal (BSFLM) from published literature, in comparison with results obtained from the current study.

	Current Study	**FAO, 2015**	**Newton *et al*., 2005**		**St-Hilaire *et al*., 2007**	**Pieterse, 2014**
Feed Substrate	Kitchen Waste	Did not specify	Pig manure	Poultry Manure	Pig Manure	Kitchen/Food Waste
Stage at Harvest	Larvae	Larvae	Prepupae	Prepupae	Prepupae	Larvae
Dry matter (% as fed)	95.4	91.30	-	-	91.60	91.30
Crude protein (%DM)	35.9	42.10	43.20	42.10	43.60	30.63
Crude fibre (%DM)	6.5	7.00	-	7.00	-	11.11
Ether extract (%DM)	48.1	26.00	28.00	34.80	33.10	44.72
Ash (%DM)	7.8	20.60	16.60	14.60	15.50	8.60
Gross energy (MJ/kg DM)	-	22.10	-	-	-	22.10

Reported ether extract content of the BSFLM ranged from 26.0% (FAO, 2015) to 44.72% (Pieterse, 2014). The ether extract content obtained in the current study (48.1%) was considerably higher when compared to the results in literature, however it compared favourably to the value achieved by Pieterse (2014) as the same processing method and feed substrate was utilized. Pieterse *et al*., 2014 reported that the time of harvest could be one of the main determining factors of ether extract and crude protein contents and, coinciding with the latter, documented results by Aniebo and Owen (2010) showed that the crude fat content of 2, 3 and 4 day-old house fly larvae increased from 22.4% to 23.9% to 27.3%, respectively, thereby clealy indicating the possible effect of age on the composition of the larvae meal. Linked with age, the stage of metamorphosis was also a significant factor influencing the chemical composition of insect meal, especially ether extract content (Chapman *et al*., 2013). As when insects reach the prepupae stage they are no longer active feeders and begin to utilize their body reserves for metamorphosis, specifically using their fat reserves as an energy source (Chapman *et al*., 2013). This phenomenon can be seen in Table 3:4, where the prepupae (Newton *et al*., 2005; St-Hilaire *et al*., 2007) had significantly lower ether extract values when compared to the larvae (Pieterse, 2014).

Table 3:4 further shows the crude fibre content of BSF larvae that ranges from 7.0% (Newton *et al*., 2005) to 11.11% (Pieterse, 2014). The crude fibre content of the larvae meal obtained in the current study (6.5%) is comparable to the value reported by Newton *et al*. (2005). Although for the current study, the stage of harvest was during the larval stage and in the study performed by Newton *et al*. (2005) the prepupae stage, the values were very similar. This could be explained by the difference in feed substrate (Newton *et al*., 1977; Pieterse, 2014) and/or a possible difference in the age at which the larvae and prepupae were collected (Calvert and Martin, 1969; Inaoka *et al*., 1999; Newton *et al*., 2005; Aniebo *et al*., 2008). The larvae may have been harvested late into the larval stage and the prepupae harvested early into the prepupae stage, but there was no clear indication of ages, which would have otherwise provided the stage of metamorphasis of the prepupae (Williams and Birt, 1972). The crude fibre content achieved in the current study is also comparable with the value reported by the FAO (2015), where the difference could again be attributed to a variation in the growth medium (Newton *et al*., 1977; Pieterse, 2014). Pieterse (2014) achieved a crude fibre content of 11.11%, which is higher than that achieved in the current study. This difference could again be attributed to the possible difference in age at harvest within the larval stage, as the chitin content

(i.e. fibre) of the insect increases with age as the insect progresses into the various stages of metamorphosis (Chapman *et al*., 2013).

Very few authors have reported on the ash content of BSFLM, however from the literature available, the ash content ranged between 8.60% (Pieterse, 2014) and 20.6% (FAO, 2015). The ash content of the larvae meal obtained in the current study (7.8%) was only comparable with that achieved by Pieterse (2014), where both values are significantly lower when compared to other reports. This again may be attributed to the fact that similar methods were employed as according to Pieterse (2014); same age at harvest and process from hatching to storage. Table 3:5 shows how the BSFLM compared to some of the other protein sources available in the animal nutrition market.

Table 3:5 Results obtained for proximate analysis of black soldier fly larvae meal, soybean meal and fish meal; and the inclusion of Sunflower oilcake meal values for comparison.

	Black soldier fly larvae meal[a]	**Soybean meal 46[a]**	**Fish meal 65[a]**	**Sunflower Oilcake meal[b]**
Dry matter (%)	95.43	89.73	89.37	89.00
Crude protein (%DM)	35.92	49.47	58.66	32.40
Crude fibre (%DM)	6.53	3.75	0.74	27.90
Ether extract (%DM)	48.09	2.07	17.17	2.20
Ash (%DM)	7.79	6.23	18.63	7.10

[a]Values as achieved in the current study for the protein sources utilized in the diet formulations.

[b]Food and Agriculture Organization (FAO), 2004.

The protein content of the larvae meal achieved in the current study (35.92%) compared well specifically with sunflower oilcake meal (32.40%), but had a significantly lower content when compared to fish meal (58.66%) and soybean oilcake meal (49.47%) when compared on a DM basis. However, the larvae meal had a substantially higher crude fat content (48.09%) when compared to the soybean oilcake meal (2.07%), fish meal (17.17%) and sunflower oilcake meal (2.20%). In terms of the crude fibre content, the larvae meal had a higher value than the soybean oilcake meal (3.70%) and the fish meal (0.74%), but the sunflower oilcake proved to have a significantly higher content when compared to all the meals with a value of 27.90%. The larvae meal had a well compared ash (mineral) content to that of the soybean (6.23%) and sunflower (7.10%) oilcake meals, but fish meal had the highest value of 18.63%.

The amino acid composition of the BSFLM is presented in Table 3:6, as the sole purpose of this study was to evaluate larvae meal as an alternative protein source in piglet diets. Thus, the respective table shows how the amino acid composition of the larvae meal in the current study compared with that of other protein sources and to the results as achieved by Pieterse (2014). There is limited information published on the amino acid digestibility of *H. illucens* larvae in pig diets, however, there have been reports on the nitrogen digestibility of the BSFLM by Newton *et al*. (1977). The results achieved, taking into the account urinary and faecal losses, show that the potential nitrogen digestibility of BSFLM is 76.0% (Newton *et al*., 1977).

Table 3:6 Results achieved for the amino acid composition of *H. illucens* larvae meal; and the inclusion of results from literature for the larvae meal and other protein sources for comparison (g/100g).

	Current study	Black soldier fly larvae meal[a]	Fish meal[b]	Soya oilcake meal[b]	Sunflower Oilcake meal[b]
Amino acids					
Alanine	1.77	2.05	4.60	2.28	1.39
Arginine	1.93	2.38	4.37	3.83	2.62
Aspartic acid	2.43	3.19	6.56	5.85	2.85
Cysteine	-	-	0.60	0.78	0.55
Glutamic acid	2.93	3.74	9.50	9.17	6.12
Glycine	1.38	1.99	4.45	2.18	1.81
Histidine*	1.01	1.23	1.66	1.35	0.78
Isoleucine*	1.25	1.62	3.24	2.38	1.33
Leucine*	1.87	2.48	5.28	3.89	2.01
Lysine*	1.78	2.41	5.66	3.16	1.13
Methionine*	0.52	0.69	2.11	0.73	0.75
Phenylalanine*	1.22	1.52	2.87	2.59	1.43
Proline	1.45	1.91	2.87	2.54	1.36
Serine	1.22	1.62	3.02	2.59	1.36
Threonine*	1.20	1.51	3.09	2.02	1.17
Tryptophan*	-	-	0.83	0.67	0.42
Tyrosine	1.75	2.27	2.19	1.81	0.75
Valine*	1.53	2.03	3.69	2.49	1.59

[a]Pieterse, 2014.
[b]Food and Agriculture Organization (FAO), 2004.
*Essential amino acids.

Table 3:7 illustrates how the various protein sources compare to the ideal amino acid profile of nursery piglets, where the amino acid profiles were determined by expressing all the indispensable amino acids as a percentage of lysine. It is seen in Table 3:7 that the BSFLM was comparable with the other protein sources and, more importantly, with the ideal amino acid profile. The values achieved in the current study were best compared with the results reported by Pieterse (2014), as this would be expected as the same feed substrate, stage of harvest and processing method was administered. The current study's results were also comparable to the ideal amino acid profile for nursery piglets as reported by the PIC Nutritional Specifications (2008), as the ratio of the amino acids to lysine were greater than that required by the animals for normal growth and development. The sunflower oilcake meal had one of the best amino acid profiles of the protein sources, but phytate present in the oilcake meal could lead to a decrease in the bioavailability of the amino acids (Thompson and Serraino, 1986). Thompson and Serraino (1986) reported that within the animals' stomach, the complex that is formed between phytate, the proteolytic enzymes and proteins could lead to a decrease in amino acid and protein digestibilities. This decrease in absorption can, however, be counteracted with the addition of microbial phytase in the diet (Thompson and Serraino, 1986).

Table 3:7 Calculated amino acid to lysine ratios in comparison to the ideal amino acid profile for nursery (3.6 – 22.7 kg) pigs.

Amino acid	Current study	Black soldier fly larvae meal[a]	Fish meal[b]	Soya oilcake meal[b]	Sunflower Oilcake meal[b]	Ideal Amino Acid Profile[c]
Lysine	100	100	100	100	100	100
Methionine + Cysteine	-	-	48.00	47.54	114.29	58
Threonine	67.42	62.66	54.67	63.93	102.86	60
Isoleucine	70.22	67.22	57.33	75.41	117.14	55
Valine	85.96	84.23	65.33	78.69	140.00	65

[a]Pieterse *et al*., 2015.
[b]Food and Agriculture Organization (FAO), 2004.
[c]Ideal amino acid profile as according to the PIC Nutritional Specifications, 2008.

3.4.2 Production parameters

Table 3:8 summarizes the results achieved during the piglet growth performance trial for the litter average piglet. There were no significant differences (P>0.05) observed between the two treatment diets in live weight, average daily gains (ADG), feed intake between weighing periods and cumulative feed intakes. However, there were some significant differences when the treatment diets were compared to the commercial feed that was utilized by the farmer. This commercial feed (Diet 3) is simply included for comparison of the live weight values and ADGs and did not form part of the feed experimental trial as it was not of a similar nutritional value nor were the animals blocked accordingly. What is of importance is to note that the overall performance of the experimental piglets (for all diets) were within the normal range experienced within a commercial scenario in South Africa; this gives credibility to the data generated by this investigation. The feed conversion ratio (FCR) was not calculated as its value would have been distorted by the milk intake and would not reflect a true indication as to the efficiency at which the feed was utilized by the piglets.

Table 3:8 Averages (± standard error) of live weight (kg), average daily gain (kg/day), feed intake (kg) and cumulative feed intake (kg) of the litter average piglet.

	Diet		
Production Parameters	**Control**	**BSFLM[1] Inclusion**	**Diet 3[2]**
Number of litters	**14**	**14**	**10**
Average number of piglets per litter	**11**	**11**	**10**
Birth			
Live Weight	1.497[a] ±0.29	1.354[a] ±0.25	1.458[a] ±0.19
Day 10 (Feed supplied)			
Live Weight	2.992[ab] ±0.44	2.846[a] ±0.51	3.122[b] ±0.42
ADG (Day 1-10)	0.136[a] ±0.01	0.130[a] ±0.01	0.151[b] ±0.02
Day 19 (Mid-way)			
Live Weight	4.792[a] ±0.62	4.651[a] ±0.83	4.817[a] ±0.70
ADG (Day 10-19)	0.213[a] ±0.02	0.211[ab] ±0.02	0.202[b] ±0.03
Feed intake (Day 10-19)	0.073[a] ±0.01	0.075[a] ±0.01	
Cumulative Feed Intake (Day 10-19)	0.073[a] ±0.02	0.075[a] ±0.02	
Day 27 (End of trial)			
Live Weight	6.737[a] ±0.73	6.579[ab] ±0.79	6.312[b] ±0.97
ADG (Day 19-27)	0.261[a] ±0.02	0.254[a] ±0.01	0.200[b] ±0.04
Feed intake (Day 19-27)	0.203[a] ±0.03	0.207[a] ±0.04	
Cumulative Feed Intake (Day 10-27)	0.276 [a] ±0.03	0.282 [a] ±0.03	
Over trial			
ADG (Day 1-27)	0.203[a] ±0.02	0.199[a] ±0.01	0.185[b] ±0.03

[a-b] Means within a row with different superscripts differ (P<0.05).

[1]BSFLM = black soldier fly larvae meal.

[2]Diet 3 is the commercial feed utilized by the farmer, included for comparison.

It can be seen from Table 3:8 that at birth there were no significant differences (P>0.05) in the average live weight values between the control and inclusion diets.

At 10 days of age the piglets were exposed to feed and the first 2 days (feed adaption period), after feed supplementation, the intakes were relatively low as piglets played with and learnt of the feed in the trough. Consumption increased from 12 days of age as piglets began to readily ingest the feed and intake values showed an expected linear increase until the end of the trial. There was no significant differences between the control and inclusion groups of piglets as pertaining to their mean weight. Interestingly enough, although the commercial diet did not form part of the feed trial, there was a significant (P<0.05) difference between the BSFLM inclusion and commercial diet. This phenomena could be correlated with the fact that the average number of piglets per litter for the commercial feed was 10 piglets and 11 piglets for the experimental feed. As litters with a lower number of piglets have been associated with larger mass gains due to lower competition experienced by the piglets for both milk and feed consumption (Bergstrom *et al*., 2015). Furthermore, the chemical composition of the commercial diet differed from that of the experimental diets.

At 19 days of age (mid-way through the feeding trial), there was no significant difference between the two treatment groups of piglets in terms of the average live weight, ADGs and intakes. The control diet had an average intake value of 0.073 kg/piglet (0.0081 g/piglet/day) and the inclusion diet a value of 0.075 kg/piglet (0.0083 g/piglet/day) from 10 to 19 days of age (Table 3:8)

At the end of the trial (27 days of age), there were no significant differences between the treatment diets in terms average live weights, ADGs and feed intakes. The ADG per piglet over the entire feed trial for the control and inclusion diets were 0.203 kg and 0.199 kg respectively, which were considerably low when compared to 377 g/piglet as reported by Bruininx *et al*. (2002). This could be explained by the exceptionally low cumulative feed intake values of 276 g/piglet and 282 g/piglet for the control and inclusion diets (Table 3:8), respectively. Pajor *et al*. (1991) reported that total feed intake from 10 to 28 days could range from anything between 118 and 1385 g/piglet. With the values achieved in the current study falling closest to the bottlom of this range, the low ADGs could be explained.

The low feed intake values could be explained by the fact that multiple feedings per day (4 times a day) were administered and that the feed troughs were too large for the amount of feed supplied at each feeding. Thus, containers were fitted into the troughs to allow ease of access of piglets to the feed. The fitting of the containers to concentrate the feed into a reachable area did, however, reduce the feeding space per animal. This, along with the problem of the piglets believing the container to be a toy for their entertainment, could explain for the low intake levels. Furthermore, the treatment diets were tested for palatability (smelt and tasted) and appeared to be odourless and bland in taste which could have had an adding effect to the low consumption levels. Figure 3:1 provides a visual representation of the effects on the average live weights for both the litter average piglet and selected piglet caused by the treatment over time (age). Both treatment diets showing the same general trend throughout the feed trial.

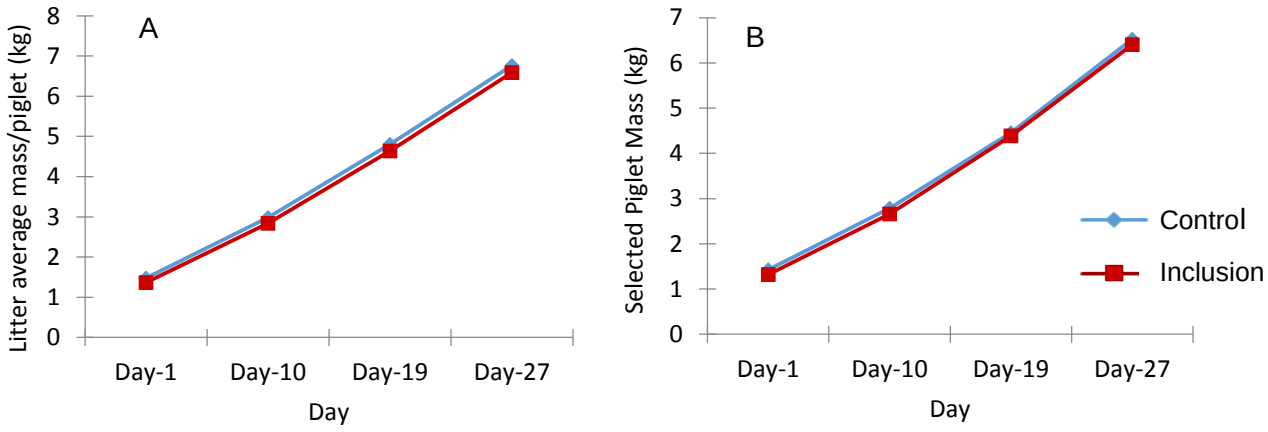

Figure 3:1 Least square means for the live weights for the litter average piglet (A) and selected piglet (B) caused by treatment over time(age) (P>0.05).

Table 3:9 summarizes the results achieved during the piglet growth performance trial for the selected piglet. There were no significant differences (P>0.05) observed between the two treatment diets in live weight and average daily gains (ADG).

Table 3:9 Averages (± standard error) of live weight (kg) and average daily gain (kg/day) of the selected piglet.

	Diet	
Production Parameters	**Control**	**BSFLM Inclusion**
Birth		
Live Weight	1.426[a] ±0.29	1.319[a] ±0.25
Day 10 (Feed supplied)		
Live Weight	2.783[a] ±0.57	2.656[a] ±0.42
ADG (Day 1-10)	0.123[a] ±0.03	0.122[a] ±0.02
Day 19 (Mid-way)		
Live Weight	4.454[a] ±1.09	4.383[a] ±0.71
ADG (Day 10-19)	0.196[a] ±0.06	0.202[a] ±0.03
Day 27 (End of trial)		
Live Weight	6.526[a] ±1.43	6.400[a] ±1.03
ADG (Day 19-27)	0.278[a] ±0.04	0.272[a] ±0.04
Over trial		
ADG (Day 1-27)	0.199[a] ±0.04	0.198[a] ±0.03

[a-b] Means within a row with different superscripts differ (P<0.05).

BSFLM = Black soldier fly larvae meal.

Interestingly enough, if the values achieved for the selected piglet (Table 3:9) were to be compared with the values for the litter average piglet (Table 3:8) for each treatment diet respectively, there was no significant difference (P>0.05). The selected piglets had higher values for ADG from day 19 to 27, but the ADGs over the entire feed trial were similar when compared to the litter average piglet. These lower values could be associated with the stress experienced by the animal when the individual data was collected from these selected piglets as a representative of their respective litters. Figure 3:2 provides a visual representation of the effects on the ADGs for both the litter average piglet and selected piglet caused by the treatment over time (age). Where both treatments diets had similar general trends throughout the feed trial with no significant differences.

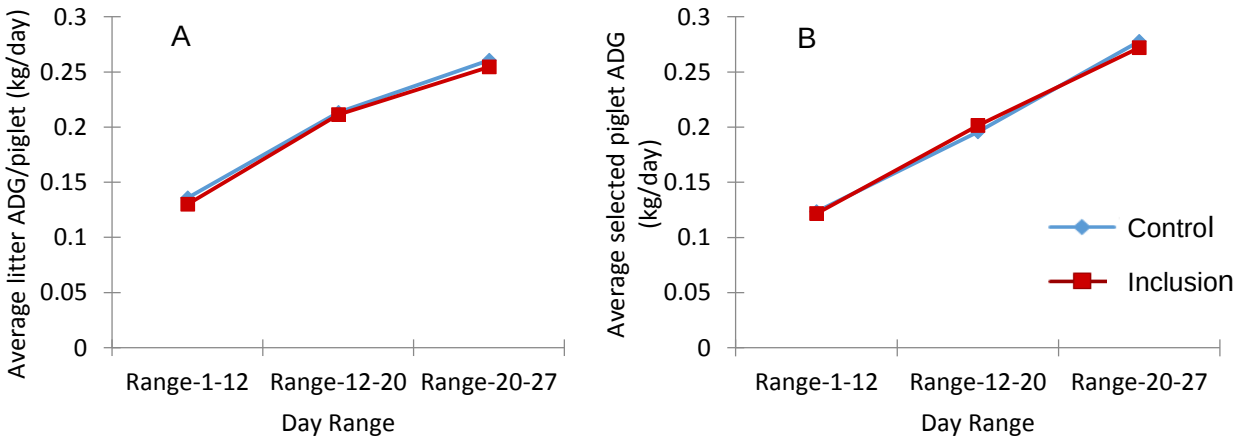

Figure 3:2 Least square means for the ADGs for the litter average piglet (A) and selected piglet (B) caused by treatment over time (age) (P>0.05).

Additionally, Figure 3:3 provides a visual representation of the effects on the litter average piglet live weights caused by age and parity interaction where it can be seen that the order for highest to lowest live weights was parity 4, 3, 6 and then 5, respectively. This provides an indication that the performance of the piglets may have decreased with an increase in parity, although there was no significant difference between interactions. Miller *et al*. (2013) reported that sow parity had a significant influence on the piglets' performance in terms of birthweight, ADGs (weaning weights) and passive immunity. As gilts (parity 1) and older sows (parity >5) tended to have lower piglet performances when compared to sows of mid-parities (3 and 4) (Miller *et al*., 2013). Figure 3:4 provides a graphical representation of the effects on the litter average piglet ADGs caused by treatment and parity interaction.

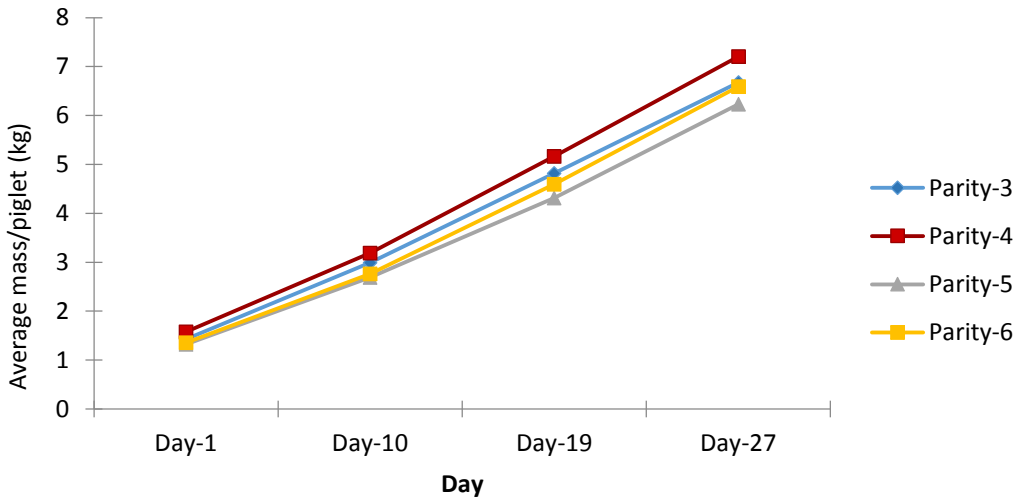

Figure 3:3 Least square means for the live weight for the litter average piglet caused by parity over time (age) (P>0.05).

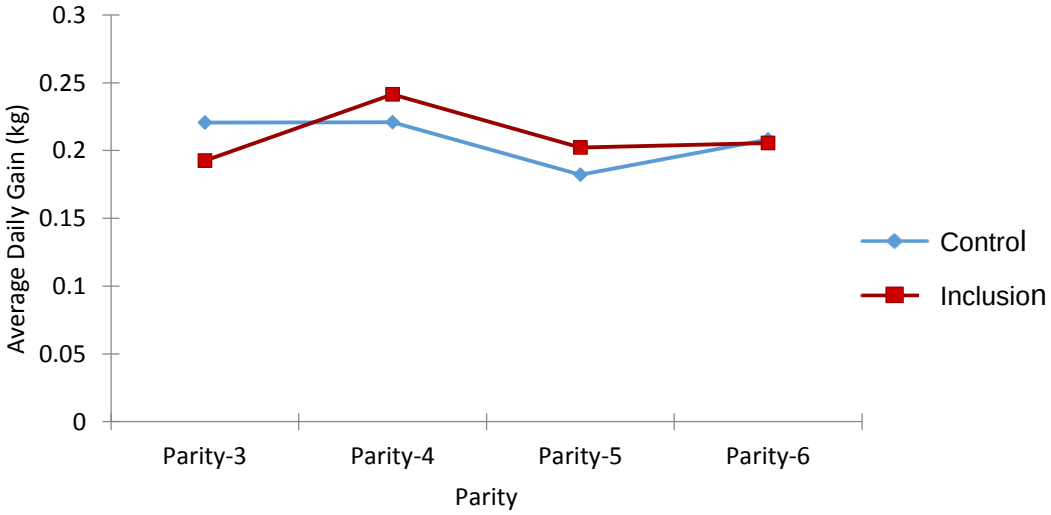

Figure 3:4 Least square means for the ADGs for the litter average piglet caused by treatment and parity interaction (P>0.05).

3.5 Conclusion

The current study showed that the proximate and amino acid values of *H. illucens* larvae meal was comparable with other traditionally used protein sources, such as fish meal, soybean oilcake meal and sunflower oilcake meal, in the animal nutrition industry. Piglets received the treatment diets from 10 days of age until weaning (27 days of age). Production parameter results showed that *H. illucens* larvae meal supplementation had no significant influence (positive or negative) on the performance of the piglets in terms of live weights, ADGs and feed intakes. The piglet intake levels were low when compared to literature (ranging from 118 - 1385 g/piglet) with only 276 g/piglet and 282 g/piglet of the control and inclusion diets being consumed over the feed trial, respectively. This could be correlated with the container adaption of the feed troughs as to allow the piglets' easy access to the feed. The containers reduced the size of the trough available to the litters for feed consumption, which may have caused dominancy issues with the weaker, smaller piglets being allowed little access to the trough. However, there was no significant variation in the size of the piglets within a litter as would be expected with the incidence of dominancy. Both of the treatment diets were also odourless and bland in taste, which may have caused a lack in palatability towards the piglets as there was no desirable smell or flavour that appealed to their senses. The same issue was experienced in the groups of piglets fed the commercial diet, which may indicate that the problem of a low feed intake may also be associated with the farming system/layout (Agostini *et al*., 2013).

Although the treatment diets were formulated for similar chemical composition, the difference in the diet ingredients and their bioavailability values between the diets may have had an influence on the performance parameters of the piglets. As piglet performance values achieved in the current study may be correlated with the difference in ingredient inclusions and not the actual inclusion of the BSFLM itself, where its inclusion was limited to only 3.5% of the total diet as to meet the piglets requirements.

From the current study, it could be concluded from the results that BSFLM can be successfully utilized as an alternative protein source to partially replace other protein sources in the ability to sustain normal piglet performance; no differences (positive or negative) were experienced between treatment diets. However, it is recommended that further studies be conducted before BSFLM is considered as a viable protein source for its use within the pig industry. The creep feed intakes were notoriously low, therefore evaluation studies should be done post-weaning in order to have larger intakes and the absence of sow's milk as a confounding factor.

3.6 References

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Chapter 4

The effect of *Hermetia illucens* (black soldier fly) larvae on the blood haematology and biochemistry of piglets.

4.1 Abstract

The effect of black soldier fly (BSF), *Hermetia illucens,* larvae meal supplementation on piglet blood parameters of 28 litters was investigated utilizing a block design consisting of two treatments (a control diet with 0% larvae meal inclusion and an inclusion diet with 3.5% larvae meal of the total volume). Black soldier fly larvae meal (BSFLM) supplementation in a four-week phase-over feeding scheme achieved results that showed no significant differences (P>0.05) between the inclusion diet when compared to the control diet in terms of haematological (Complete Blood Count) and biochemical (albumin, calcium and corrected calcium, phosphorus, iron and immunoglobulin concentration) parameters; reflecting health status (immunology) and/or mineral bioavailability. Blood sample collection mediated stress and dilution effect was encountered during data collection, as well as the unintended administration of antibiotics. It can, however, be concluded from this trial that BSFLM can be utilized as an alternative protein source to partially replace other protein sources with no adverse effects on blood metabolite concentrations of pre-weaned piglets (trial conducted from 1 to 28 days of age).

Keywords: blood, haematology, biochemistry, BSF, larvae meal, pig, diet

4.2 Introduction

The haematological and biochemical parameters of pigs are influenced by a variety of environmental and physiological factors, which include stress (Minka and Ayo, 2007), diet (Etim *et al*., 2014), season (Chmielowiec-Korzeniowska *et al*., 2012) and physiological factors, such as genetics, age, sex and physiological stage (Tumbleson and Scholl, 1981). Advances in technology have allowed for dramatic changes in the production of pigs, as well as improvements in the laboratory techniques used to accurately quantify blood parameters (Wilson *et al*., 1972). Blood profiling of random animals within a farming system could provide a valuable indication of the clinical health of the overall herd, where parameters are strongly influenced by diseases and nutritional deficiencies (Friendship *et al*., 1984). In the case of the detection of certain trace element deficiencies, measuring the activity of dependent enzymes provides an indication as to what deficiencies are present (Mills, 1974). This provides an early warning signal for farmers to alter the diets of the animals to ensure that all the nutritional requirements of the animals, for both maintenance and production, are fully satisfied (Church *et al*., 1984; Maxwell *et al*., 1990; Etim *et al*., 2014). However, herd haematological and biochemical profiles have only been rarely utilized in the pig veterinary practise and, for this reason, future studies involving problem herds are warranted in order to assess the value of such a diagnostic technique, though it may come at a high financial expense (Friendship *et al*., 1984).

Biochemical parameters offer as a good indication as to the true digestibility and chemical availability of minerals, as well as to what effect diet ingredients have on the physiological status of the animal

(Schiavon *et al*., 2000; Bangert *et al*., 2008). Black soldier fly larvae meal (BSFLM) is a new, sustainable protein source in animal nutrition and its protein content ranges from 30.63 to 43.60% and its ether extract content from 26.0 to 44.72%. It has significantly higher concentrations of calcium and iron when compared to other protein sources, such as fish meal, soybean oilcake meal and sunflower oilcake meal, as seen in Table 4:1. Thus, it was worth investigating whether these large concentrations in the BSFLM are easily available for absorption and utilization by the animal.

Table 4:1 Comparison between the nutritional composition of black soldier fly larvae meal and other traditionally used protein sources.

		Black soldier fly larvae meal[a]	Fish meal[b]	Soya oilcake meal[b]	Sunflower Oilcake meal[b]
Main analysis	**Unit**				
Dry matter	% as fed	91.3	92.1	87.9	89
Crude protein	% DM	42.1	75.4	51.8	32.4
Crude fibre	% DM	7	-	6.7	27.9
Ether extract	% DM	26	11	2	2.2
Ash	% DM	20.6	13.6	7.1	7.1
Gross energy	MJ/kg DM	22.1	21.9	19.7	19.4
Minerals					
Calcium	g/kg DM	75.6	26.5	3.9	4.4
Phosphorus	g/kg DM	9	22.3	6.9	11.6
Potassium	g/kg DM	6.9	11.9	23.7	16.9
Sodium	g/kg DM	1.3	10.9	0.1	0.1
Magnesium	g/kg DM	3.9	3.1	3.1	5.6
Manganese	mg/kg DM	246	10	45	38
Zinc	mg/kg DM	108	99	54	96
Copper	mg/kg DM	6	-	18	32
Iron	mg/kg DM	1370	-	346	271

[a]Food and Agriculture Organization (FAO), 2004.

[b]Food and Agriculture Organization (FAO), 2015.

Further, the inclusion of BSFLM in broiler diets has been reported to decrease the incidence of metabolic skeletal disorders and improve the overall health of the birds (Pieterse, 2014; Pieterse *et al*., 2014; Uushona, 2015). Thus, this warranted for research to be conducted into pig production to detect whether or not a similar phenomenon would be discovered and to what impact this possible improvement in production has on blood parameters. The immunological status of an animal has been reported to be a function of leukocytes or white blood cells (WBCs), as they play a crucial role by producing immunoglobulins or antibodies that bind to bacteria or viruses and aid in their destruction (Litman *et al*., 1993). Lymphocytes, a type of WBC, play a primary role in the immune system of both human and animals (Ameen *et al*., 2007).

Despite the availability of BSFLM in South Africa and the need for alternative protein sources in the animal production industry, it has not yet been used as a feed ingredient in piglet diets. Thus, the objective of this study was to investigate the effects of BSFLM as a protein source on piglet blood parameters, specifically immunological and mineral bioavailability characteristics.

4.3 Research design and methodology

4.3.1 Animals and diets

For this trial, the three hundred and fifteen piglets that were among the 28 litters utilized for the production trial were utlilized, therefore the animals were managed in the exact same manner as described in Chapter 3 (animal selection, housing, experimental diets, etc.).

The same piglet that was utilized for the collection of individual data in Chapter 3 was used for blood collection, so as mentioned, the litter average mass per piglet was calculated and the single piglet that was within the closest range to this average was selected for the collection of individual data, as a representation of that litter. Thus, 28 piglets (14 per treatment) were utilized for blood sampling for this specific trial.

4.3.2 Experimental design and trial procedure

Three hundred and fifteen piglets were among the 28 litters utilized for the blood parameter trial, where each litter was with its respective sow in the farrowing pen. The 28 litters were placed into two blocks according to sows' farrowing dates. The two treatments were administered with eight replications per treatment with an average of 11 piglets per replicate for block 1 and six replications per treatment with average of 11 piglets per replicate for block 2. The average piglet mass for each litter was calculated (total litter mass divided by the number of piglets in the litter) and the piglet that fell closest to this average was selected for the collection of individual data as a representation for its respective litter.

4.3.3 Data collection and analyses

Blood withdrawals were performed at 9 (before treatment diets were administered), 18 (middle of feeding scheme) and 26 days of age (end of trial). The veterinary services generously offered by the Elsenburg experimental farm, Western Cape were utilized for blood collection.

NOTE: In the current trial, great difficulty was experienced by the Animal Technician at each withdrawal period, where in the attempt to withdraw blood samples from the animals there were multiple punctures required for sample collection. This also leading to the prolonged handling of each piglet, which in respect put a great amount of stress on the animal. Initially the use of a 21 gauge needle and syringe was to be utilized and blood injected into the microtainer tubes, but due to difficulty of withdrawal, the Animal Technician made an onsite decision to use 4 mL vacutainer tubes and to transfer the blood samples from these tubes to the microtainer tubes. The Animal Technician stated that there should not be any significant influences on the blood cell count. Animals also received an iron injection at 3 days of age (normal farming practises).

Assumptions

I. Normality between the piglets within a litter.

II. Normality between the litters.

4.3.3.1 Samples collected in EDTA

Samples were collected utilizing 21 gauge needles and 4 mL K_3EDTA vacutainer tubes and blood was transferred to two 0.5 mL K_3EDTA microtainer tubes, as to achieve duplicate samples for testing (Method adopted by Animal Technician). Samples were placed onto ice until testing was conducted, which was achieved within 5 h after withdrawal before any deterioration was to occur. Tests were performed utilizing a Cell-Dyn 3700 Haematology Analyzer (Abbott Diagnostics, USA) at the Animal Science department, Stellenbosch University, Western Cape, South Africa. These tests included the counts for White Blood Cells (WBCs) and differential WBCs (Neutrophils, Lymphocytes, Monocytes, Eosinophils and Basophils), Red Blood Cells (RBCs), Haemoglobin (HGB), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Blood Cell Distribution Width (RDW), Platelets (PLT) and Mean Platelet Volume (MPV).

Cell-Dyn 3700 Haematology Analyzer protocol

The mode of aspiration was done by means of the Open Sampler Mode, which was used to aspirate the sample from a collection tube that had been opened and held under the Open Sample Aspiration Probe. The aspiration volume for this particular mode was 130 µL ± 5%, where the sample was aspirated into the Analyzer by the Aspiration Peristaltic Pump, through the Shear Valve.

The white blood cell (WBC) analysis was performed by taking two measurements, namely the WBC optical count (WOC) and WBC impedance count (WIC). The WOC Sheath Syringe dispensed 1.6 mL of sheath reagent through the Shear Valve, picking up 32 µL of the sample. The sample segment and sheath were then routed to the Mixing Chamber where the dilution was bubble mixed with a final dilution of 1:51. The Peristaltic Pump transfered the dilution from the Mixing Chamber to the Sample Feed Nozzle in the Flow Cell and a stream of sheath reagent was directed through the cell. A metering syringe injected 78 µL of the dilution into the Flow Cell-sheath stream and a laser beam was focused on the Flow Cell and as the sample stream intersected the laser beam, the light scattered by the cells was measured for different angular intervals.

The WIC Diluent Syringe dispensed 5.25 mL of diluent through the Shear Valve, picking up 20 µL of the sample segment. The segment and diluent was routed to the Mixing Chamber in the von Beherns WIC/HGB Transducer and the Lyse Syringe delivered 0.75 mL of the Lyse to the Mixing Chamber simultaneously. The dilution was bubble-mixed (final dilution is 1:301) and pulled through the aperture by vacuum, where the Volumetric Meter ensured that 200 µL was utilized for measurement. The Electrical Impedance was then used to count the WBCs as the dilution passed through the aperture. The remaining dilution was transferred to the Haemaglobin (HGB) Flow Cell and the HGB concentration was measured spectrophotometrically.

The red blood cell (RBC) and platelet (PLT) analysis were also performed, where the diluent syringe dispensed 7.2 mL of the diluent through a Shear Valve, picking up 0.74 µL of the sample segment. The sample segment and diluent was routed to the Mixing Chamber of von Behrens RBC/PLT Transducer where the dilution was bubble-mixed (final dilution is 1:9760). The dilution was pulled through the aperture by vacuum and the Volumetric Meter ensured that 100 µL of the dilution was measured. An Electrical Impedance was used to count the RBCs and PLTs as they passed through the aperture.

4.3.3.2 Samples collected in serum

Samples were collected utilizing 21 gauge needles and 4 mL Serum vacutainer tubes and blood was transferred to two 0.6 mL Serum microtainer tubes, as to achieve duplicate samples for testing. Samples were placed onto ice and centrifuged within 5 h at 3500 rpm at 4°C for 10 min utilizing a Spectafuge 24D (Labnet) centrifuge. The serum was separated by pipet from other blood components and frozen in 0.5 mL Eppendorf tubes at -20°C within 5 h after withdrawal before any deterioration of blood samples was to occur. Tests were performed utilizing a Siemens Advia 2120 Analyzer at the Haematology department, Tygerberg, National Health Laboratory Service (NHLS), Western Cape, South Africa. These tests included the concentrations of albumin, calcium, phosphorus and iron, as well as the concentration of the major serum constituents of antibodies which are IgA, IgG, and IgM. Albumin was tested as to calculate the corrected calcium concentration of the blood samples.

Note: The Siemens Advia 2120 Analyzer is calibrated specifically for human blood samples, however, piglet samples were tested by before the onset of the trial. Duplicate samples were tested by the analyser and then also microscopically, and proved to provide accurate results.

Siemens Advia 2120 Analyzer protocol

I. Calcium

The calcium ions form a coloured complex with Arsenazo III, which was measured at 658/694 nm. The amount of calcium present in the sample was directly proportional to the intensity of the coloured complex formed.

Equation 4:1

$$Ca^{2+} + \text{Arsenazo III} \xrightarrow{pH\,5.9} \text{Ca-Arsenazo III Complex (purple)}$$

II. Albumin

Serum or plasma albumin quantitatively binds to Bromocresol Green (BCG) to form an albumin-BCG complex that was measured as an endpoint reaction at 596/694 nm.

Equation 4:2

$$\text{BCG} + \text{Albumin} \xrightarrow{pH\,4.2} \text{BCG Albumin complex}$$

III. Corrected Calcium

The corrected calcium was a calculated value based on the total measure of calcium (mmol/L) and albumin (g/L). The equation was as taken from Burtis *et al.* (2012).

Equation 4:3

$$\text{Corrected calcium (mmol/L)} = 0.02\,[40 - \text{Albumin (g/L)}] + \text{Calcium (mmol/L)}$$

IV. Phosphorus

Inorganic phosphorus reacted with ammonium molybdate in the presence of sulfuric acid to form an unreduced phosphomolybdate complex, which was measured as an endpoint reaction at 340/658 nm.

Equation 4:4

$$\text{Phosphate} + \text{Molybdate} \xrightarrow{H^+} \text{Phosphomolybdate complex}$$

V. Iron

Ferric iron was separated from its carrier protein, transferrin, in an acid medium and simultaneously reduced to the ferrous form. The ferrous iron was then complexed with ferrozine, a sensitive iron indicator, to produce a coloured chromophore, which absorbs at 571/658 nm.

Equation 4:5

$$\text{Transferrin } (Fe^{3+}) \xrightarrow{H^+} \text{Apotransferrin} + Fe^{3+}$$

$$Fe^{3+} + \text{Ascorbic Acid} \longrightarrow Fe^{2+}$$

$$Fe^{2+} + \text{Ferrozine} \longrightarrow Fe^{2+}/\text{Ferrozine Complex}$$

VI. Immunoglobulins

A PEG-enhanced immunoturbidimetric method was used for blood IgM and IgA, where they were diluted and then reacted with a specific antiserum to form a precipitate that can be measured turbidimetrically at 340/694 nm. A standard curve was constructed for each from the absorbencies of standards and the concentration of both IgM and IGA was determined.

IgG was determined utilizing a PEG-enhanced immunoturbidimetric method, where it was diluted and then reacted with a specific antiserum to form a precipitate that can be measured turbidimetrically at 340/694 nm. A calibration curve was constructed from the absorbencies of calibrators and the concentration of IgG was determined.

4.3.3.3 Statistical analysis

As there were a number of variables experienced when recording the data, an exploratory analyses was done to determine whether or not their influence was sufficient to warrant their inclusion in the final model for analyses.

The exploratory analyses included fitting the full model and combination of variables using the PROC GLM of SAS for Windows Version 9.3 (statistical software). A probability of $P<0.05$ was used to determine significance. The statistics were done using analysis of covariance with least square means (LSM) calculated with Bonferroni *post hoc* test. First, a full model was analysed with the main factor of parities being tested, with farrowing dates included as blocks. Age (included as blood sampling periods) and number of piglets per litter were also included as co-variables in the different models used for analysis. Due to parities, blocks and number of piglets per litter having no significant effects, these were excluded from the models. Breed was also excluded due to the very few Landrace animals included in the experiment.

After exclusion of all these variables, the final model included the variables of age (included as blood sampling period) and treatment. The piglet blood metabolite concentrations were analysed by means of PROC GLM for treatment with each withdrawal period being analysed separately. Descriptive statistics and graphs of treatments and age interaction for each blood metabolite are included to provide a graphical representation of the change in concentrations over time.

4.4 Results and discussion

4.4.1 Blood haematological parameters

Table 4:3 summarizes the blood haematology results achieved during the piglet blood trial. There were no treatment differences (P>0.05) observed at each sampling period, except for that of MCV and MCH, where the BSFLM diet showed higher values. This may be related to the initial high values of MCV and MCH for the BSFLM diet at the first withdrawal, where Figure 4:1 shows that the two dietary treatments were similar for each blood sampling period. If either the treatment diets' starting or intercept points for sampling period 1 were held constant, then there would be no significant difference between the control and BSFLM inclusion diets.

There were significant differences (P<0.05) observed in some of the parameters between sampling periods, thus indicating a change in the blood counts over time. This phenomenon is normal in growing and developing piglets (Tumbleson and Scholl, 1981). If compared to haematological reference values in literature, most of the counts fell within normal reference intervals, except those for monocytes, basophils and platelets which all fell above the normal intervals. This could be explained by the dilution effect of transferring the samples from one tube to another as the ratio of blood to additive is altered (Banfi *et al*., 2007), and/or sample collection mediated stress (Epstein *et al*., 1988; Dubreuil *et al*., 1990) and/or variation in analysers utilized (Lippi *et al*., 1989).

EDTA cannot completely stabilize platelets, which may have allowed for some morphological alterations to occur. When platelets come in contact with the EDTA additive, they undergo a time-dependent change from a discoidal to a spherical shape (Karpatkin *et al*., 1978). When the platelets undergo a spherical change it may lead to an apparent increase in the volume when the particle passes through the analyser (Lippi *et al*., 1989). However, this is more frequent in impedance-based analyzers rather than light-scattering-based analyzers (analyser utilized in the current study) (Lippi *et al*., 1989). In the light-scattering-based haemocytometers, in which the haematological parameters are defined by both volume and light refraction indexes, the MPV measure could be more variable (Lippi *et al*., 1989). In these systems, the MPV is usually decreased, although an increase can be recorded in up to one-third of all cases (Lippi *et al*., 1989). Bessman *et al*. (1982) reported that there is a consistent inverse association between the number and volume of platelets, such that the relationship is linear up to 400×10^9 PLTs/L. The change in MPV depends on the time of contact with the anticoagulant, where membrane permeability changes through a cyclic AMP (cAMP) -mediated reaction (Bessman *et al*., 1982). In particular, the values recorded by light scattering analyzers are slightly higher than those measured by impedance-based analyzers (Lippi *et al*., 1989).

Sampling stress can also have a significant influence and cause haematological variation, as the response to stress develops within 2 min which rapidly affects the total WBC count and the leukogram (differential WBC counts) (Dubreuil *et al*., 1990). Elevations in the haemoglobin concentration, haematocrit and RBC sedimentation rate have also been correlated with stress (Dubreuil *et al.*, 1993). Epstein *et al.* (1988) reported that venipunture should be minimally traumatic for the animal to minimize platelet activation. As a slow or traumatic venipuncture (poking around the vein or exiting the vein during sample withdrawal) can activate platelet clumping and induce small microclots within the sample or even clotting the sample completely. Microclots particularly affect the WBC, leukogram and PLT counts (Epstein *et al*., 1988). Guder *et al*. (1996) also reported that WBC and PLT counts can be affected by cryoglobulins, which aggregate when blood samples cool from

body temperature to room temperature and are falsely identified as WBCs or PLTs by the analyser. Difficult venipuncture, particularly through a small gauge needle, can also further result in the shearing of RBCs which affects cell counts and results in artificial haemolysis (Dubreuil *et al.*, 1993).

Table 4:2 Blood Haematology or Complete Blood Count (CBC) results.

	Sampling Period					
	1 (Day 9)		2 (Day 18)		3 (Day 26)	
Diet	**Control**	**BSFLM**	**Control**	**BSFLM**	**Control**	**BSFLM**
WBC (10e^9/l)	8.71 ± 3.00	6.57 ± 3.49	10.25 ± 5.78	9.22 ± 3.93	8.49 ± 3.08	11.19 ± 4.81
NEU (%)	51.40 ± 11.64	47.44 ± 12.59	46.70 ± 14.98	45.46 ± 12.15	41.27 ± 16.04	43.55 ± 14.50
LYM (%)	12.83 ± 5.93	18.94 ± 18.46	16.35 ± 18.34	15.14 ± 9.39	24.56 ± 19.64	22.98 ± 14.12
MONO (%)	17.33 ± 4.56	16.63 ± 3.95	21.23 ± 6.73	21.30 ± 6.17	18.84 ± 5.67	21.30 ± 6.39
EOS (%)	1.35 ± 0.75	1.58 ± 1.09	0.80 ± 0.62	0.98 ± 0.41	0.56 ± 0.63	0.84 ± 0.86
BASO (%)	17.09 ± 13.39	15.42 ± 9.55	14.93 ± 8.65	17.12 ± 7.27	11.24 ± 11.87	11.34 ± 8.88
RBC (10e^{12}/l)	4.67 ± 0.98	4.11 ± 1.41	5.24 ± 1.02	5.05 ± 0.51	5.78 ± 1.20	5.80 ± 0.78
HGB (g/dL)	10.11 ± 2.08	9.49 ± 3.10	10.91 ± 2.60	11.20 ± 0.79	10.91 ± 3.10	11.64 ± 1.95
HCT (%)	54.12 ± 10.58	49.95 ± 16.58	57.89 ± 13.18	59.97 ± 5.06	57.40 ± 15.07	62.01 ± 8.76
MCV (fL)	113.98 ± 15.98	121.90 ± 7.69	109.9 ± 13.15	118.96 ± 6.50	98.37 ± 13.89	107.07 ± 5.33
MCH (pg)	21.30 ± 3.48	23.30 ± 1.55	20.64 ± 2.60	22.25 ± 1.29	18.65 ± 3.27	20.06 ± 1.65
MCHC (g/dL)	18.63 ± 1.00	19.13 ± 0.73	18.76 ± 0.69	18.70 ± 0.65	18.86 ± 0.90	18.72 ± 0.93
RDW (%)	26.24 ± 7.33	25.81 ± 5.05	22.71 ± 4.64	20.53 ± 2.15	25.45 ± 8.41	18.72 ± 0.93
PLT (10e^9/L)	644 ± 281	505 ± 325	631 ± 249	585 ± 174	519 ± 274	537 ± 295
MPV (fL)	17.84 ± 5.58	15.98 ± 3.85	17.01 ± 2.80	17.34 ± 2.38	16.1 ± 3.56	14.98 ± 0.79

Abbreviations: WBC= White Blood Cells, NEU= Neutrophils, LYM= Lymphocytes, MONO= Monocytes, EOS= Eosinophils, BASO= Basophils, RBC=Red Blood Cells, HGB= Haemoglobin, HCT= Haematocrit, MCV= Mean Corpuscular Volume, MCH= Mean Corpuscular Haemoglobin, MCHC= Mean Corpuscular Haemoglobin Concentration, RDW= Red Blood cell Distribution Width, PLT= Platelets, MPV= Mean Platelet Volume, BSFLM = black soldier fly larvae meal.

There are several additional problems that have been reported to be associated with the use of EDTA as an anticoagulant, which include leukocyte clumping (Hillyer *et al*., 1990). Coincident clumping of WBCs and PLTs have been reported to occur at temperatures lower than 37°C, where PLT clumping cannot be prevented using anticoagulants other than EDTA (Epstein *et al*.,1988).

With these facts in knowledge, the high values for monocytes, basophils and platelets achieved in the current study may be explained. To what extent the dilution effect had on the results is uncertain, as most blood parameters still fell within normal reference intervals. These results, and information in literature, indicate that the effect of the sample collection mediated stress may have had a significant influence on WBC (and differential) and PLT counts as described by Epstein *et al*. (1988). The graphs in Figure 4:1 provide a visual representation for the average blood concentrations of the various haematological parameters caused by withdrawal and treatment interaction. In Figure 4:1 it can be seen that the HGB concentration and HCT percentage trend between withdrawals had a higher increasing trend for the BSFLM diet when compared to the control diet. Although there was no significant (P>0.05) difference between the two treatment diets for these specific parameters, there may be biological correlation between the BSFLM and the respective higher values as the meal has an incredibly high iron content. However, evidence of this is not clear due to low intake levels (described in Chapter 3), problems encountered during blood sampling and antibiotic administration. Therefore, further studies would be required to associate these findings with the BSFLM.

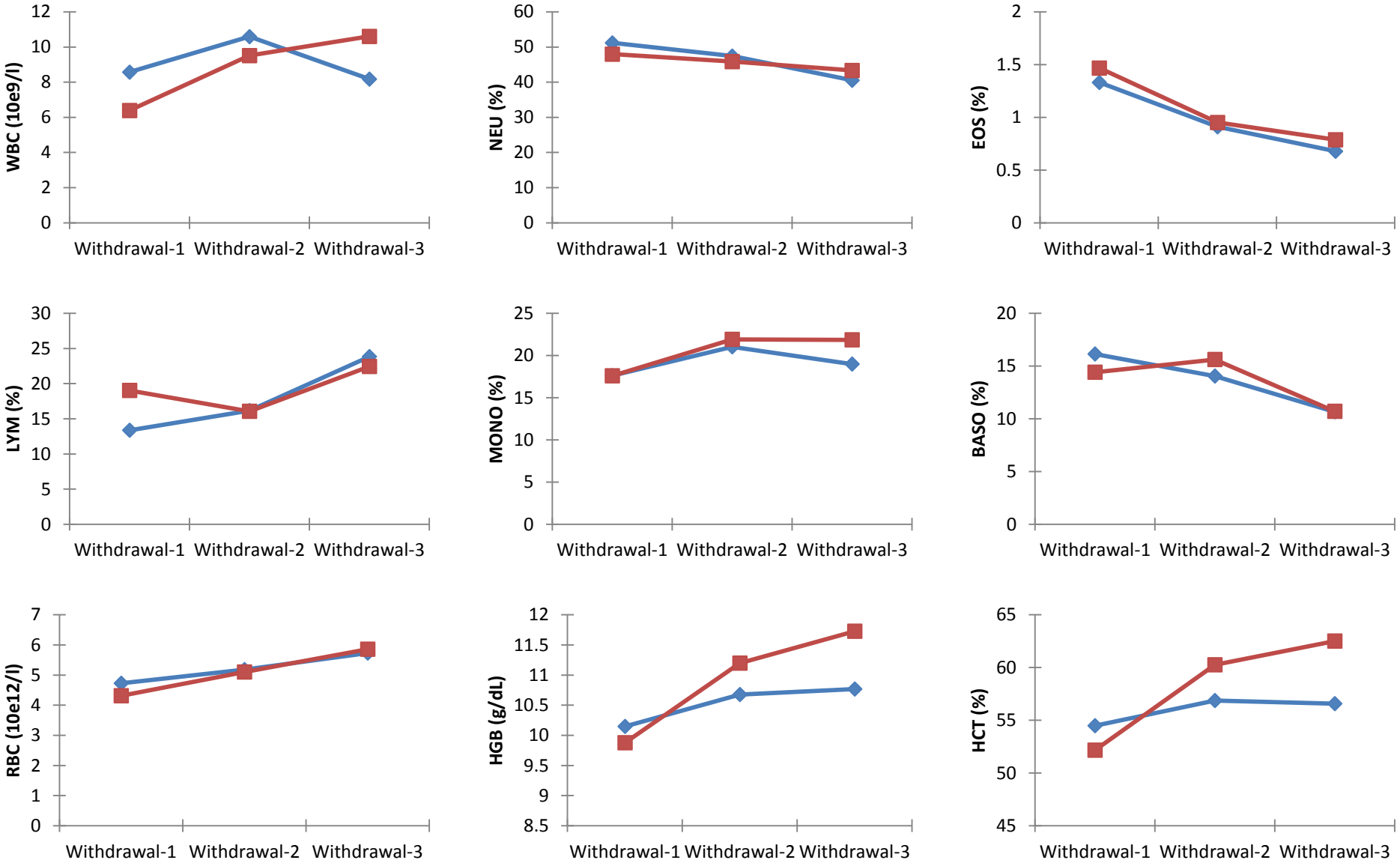
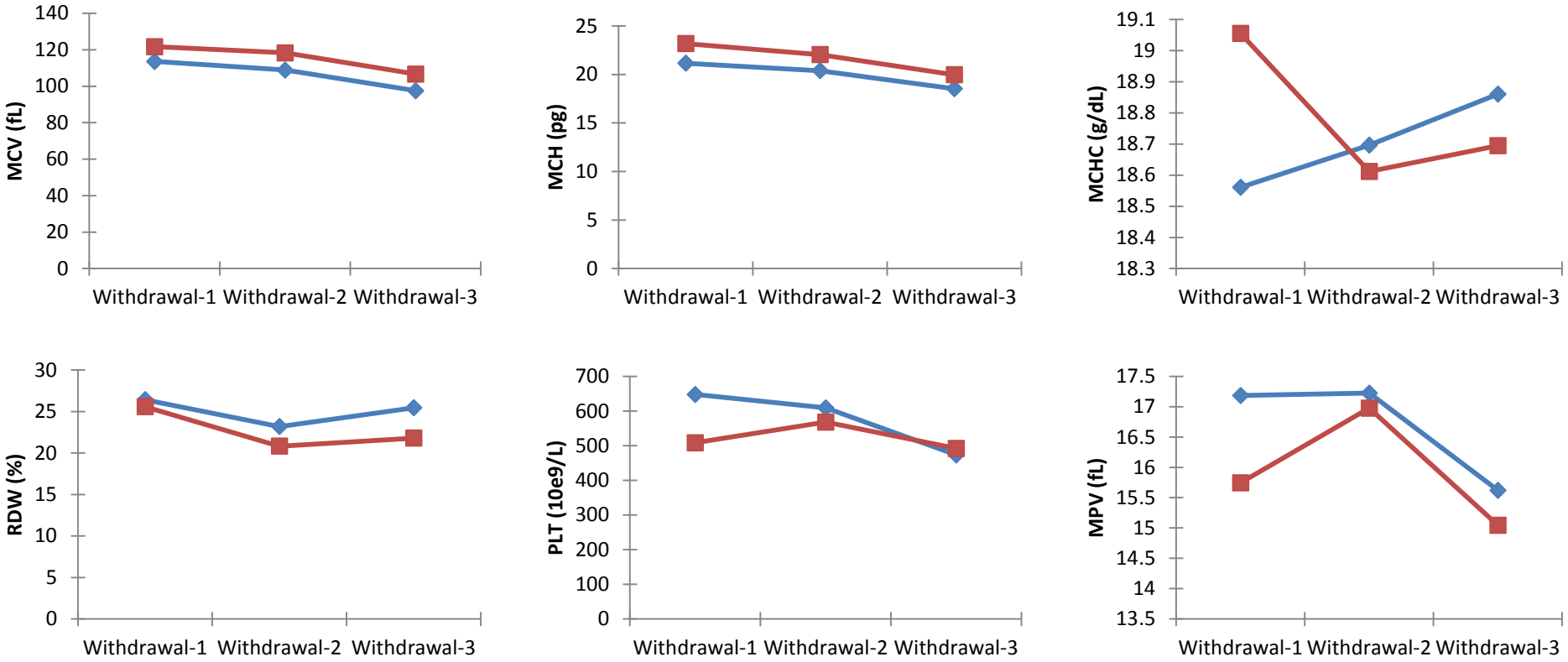


Figure 4:1 Average blood concentrations of the various haematological parameters caused by withdrawal and treatment interaction.



WBC - White Blood Cells
LYM - Lymphocytes
EOS - Eosinophils
RBC - Red Blood Cells
HCT - Haematocrit
MCH - Mean Corpuscular Haemoglobin
RDW - Red Blood cell Distribution Width
MPV - Mean Platelet Volume

NEU - Neutrophils
MONO - Monocytes
BASO - Basophils
HGB - Haemoglobin
MCV - Mean Corpuscular Volume
MCHC - Mean Corpuscular Haemoglobin Concentration
PLT – Platelets

Figure 4:1 Average blood concentrations of the various haematological parameters caused by withdrawal and treatment interaction (cont.).

4.4.2 Blood biochemical parameters

Table 4:4 summarizes the blood biochemical results achieved during the piglet performance trial. There were no treatment differences (P>0.05) observed at each blood sampling period, however there were significant differences (P<0.05) between sampling periods observed in some of the parameters, thus indicating a change in the counts over time. Once again, this phenomenon is normal in growing and developing piglets (Tumbleson and Scholl, 1981). If compared to haematological reference values in literature, all the counts were within normal reference intervals, except the concentrations of the immunoglobulins which were all lower than the normal intervals. This could again be explained by the dilution effect (Banfi *et al*., 2007), sample collection mediated stress (Epstein *et al*.,1988; Dubreuil *et al*., 1990) and/or specific analyser utilized (Lippi *et al*., 1989).

Table 4:3 Blood biochemical results.

	Sampling Period					
	1 (Day 9)		2 (Day 18)		3 (Day 26)	
Diet	**Control**	**BSFLM**	**Control**	**BSFLM**	**Control**	**BSFLM**
Calcium (mmol/l)	2.49 ± 0.38	2.39 ± 0.36	2.63 ± 0.25	2.64 ± 0.21	2.53 ± 0.18	2.47 ± 0.17
Albumin (g/l)	22.79 ± 4.96	20.50 ± 4.03	28.07 ± 3.60	27.07 ± 3.93	29.79 ± 3.33	29.00 ± 3.31
Ca -corrected (mmol/l)	2.83 ± 0.30	2.78 ± 0.33	2.87 ± 0.21	2.90 ± 0.15	2.74 ± 0.14	2.69 ± 0.13
Phosphorous (mmol/l)	2.76 ± 0.42	2.65 ± 0.38	2.80 ± 0.43	2.92 ± 0.24	2.77 ± 0.44	2.80 ± 0.18
Iron (µmol/l)	20.02 ± 9.36	22.46 ± 4.97	15.59 ± 10.30	19.44 ± 6.38	11.41 ± 8.49	13.84 ± 7.91
IgG (g/l)	5.10 ± 1.72	4.45 ± 1.97	3.22 ± 1.17	2.89 ± 1.05	2.02 ± 0.48	2.04 ± 0.70
IgA (g/l)	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
IgM (g/l)	<0.25	<0.25	0.26 ± 0.02	0.26 ± 0.02	0.35 ± 0.08	0.40 ± 0.17

Ig= immunoglobulin; BSFLM = Black soldier fly larvae meal.

Furthermore, the lower values could also be associated with the low feed intakes experienced in the current trial as described in Chapter 3, where diet composition as well as level of intake can influence blood parameters (Schiavon *et al*., 2000; Annongu and Folorunso, 2005; Fasuyi and Ibitayo, 2010). Milk intake of the piglets also plays a significant factor on blood parameters, specifically immunoglobulin concentrations (Hendrix *et al*., 1976; Inoue *et al*., 1980; Litman *et al*., 1993). In fact, a passive transfer of immunity via the intake of colostrum is incredibly important in pigs, as the epitheliochorial nature of the sow's placenta prevents the transfer of most antibodies across the placenta (Litman *et al*., 1993). IgG is the only immunoglobulin capable of crossing the placenta to give passive immunity to the foetus (Litman *et al*., 1993). Colostrum is also an important supply of IgG and its short time window (24 to 36 h) during which the piglet is able to absorb it in its intact form and transfer it the blood stream makes milk intake critically important for the first few days after birth (Litman *et al*., 1993). A new-born piglet is therefore reliant on IgG absorbed from the colostrum for humoral immune protection until its own active immune system has sufficiently matured to respond to (to produce antibodies against) foreign antigens (Hendrix *et al*., 1976). Butler *et al*. (1981) reported that no, or very low, levels of IgA and IgM are present in the piglet until about 10 days of age, thereafter IgM rather than IgA is the dominant type. The successful transfer of passive immunity relies on several important factors, which include that the milk of the sow must contain sufficient amounts of immunoglobulins (IgG before piglet gut closure and IgA and IgM post-closure), the antibodies must be delivered to the site of absorption intact and that IgG must be absorbed intact

and delivered to the circulation of the piglet (Bland and Rooke, 1998). Parity, season and genotype have also been suggested as factors that influence colostrum IgG concentrations (Inoue *et al*., 1980). Bland and Rooke (1998) reported that IgG concentrations can even vary between the different parts of the udder, as caudal teats tend to have lower concentrations than cranial teats which may cause variation in blood concentrations between piglets within a litter due to suckling hierarchy.

With all these factors, the low values for the immunoglobulins (when compared to reference values in literature) achieved in the current study could be explained. However, the concentrations of the immunoglobulins did follow the expected trend of normal developing piglets. The concentrations of IgG decreased over time and concentrations of IgM increased, which is caused by the physiological transfer from passive to active immunity in the early stages of the piglets' lives. Figure 4:2 provides a visual representation for the average blood concentrations of the various biochemical parameters caused by withdrawal and treatment interaction.

The significant decrease in iron from sampling period 1 to sampling period 3 may be explained by the supplementation of iron at three days of age. Iron is necessary to prevent anaemia in piglets, as iron deficiency anaemia can develop rapidly in nursing piglets (Clark, 2009). This may be caused by low iron reserves in the newborn piglet, the low iron in the sow's colostrum and milk, the lack of contact with iron in the soil and the rapid growth rate of the piglets (Clark, 2009). Iron is a necessary nutrient for the growth of microorganisms in the piglet's digestive tract and with no access to soil, iron deficiency anaemia may result within 7 to 10 days after birth (Clark, 2009). Thus, the supplementation of iron has become a general management practise in pig production systems to help animals achieve proper growth and development.

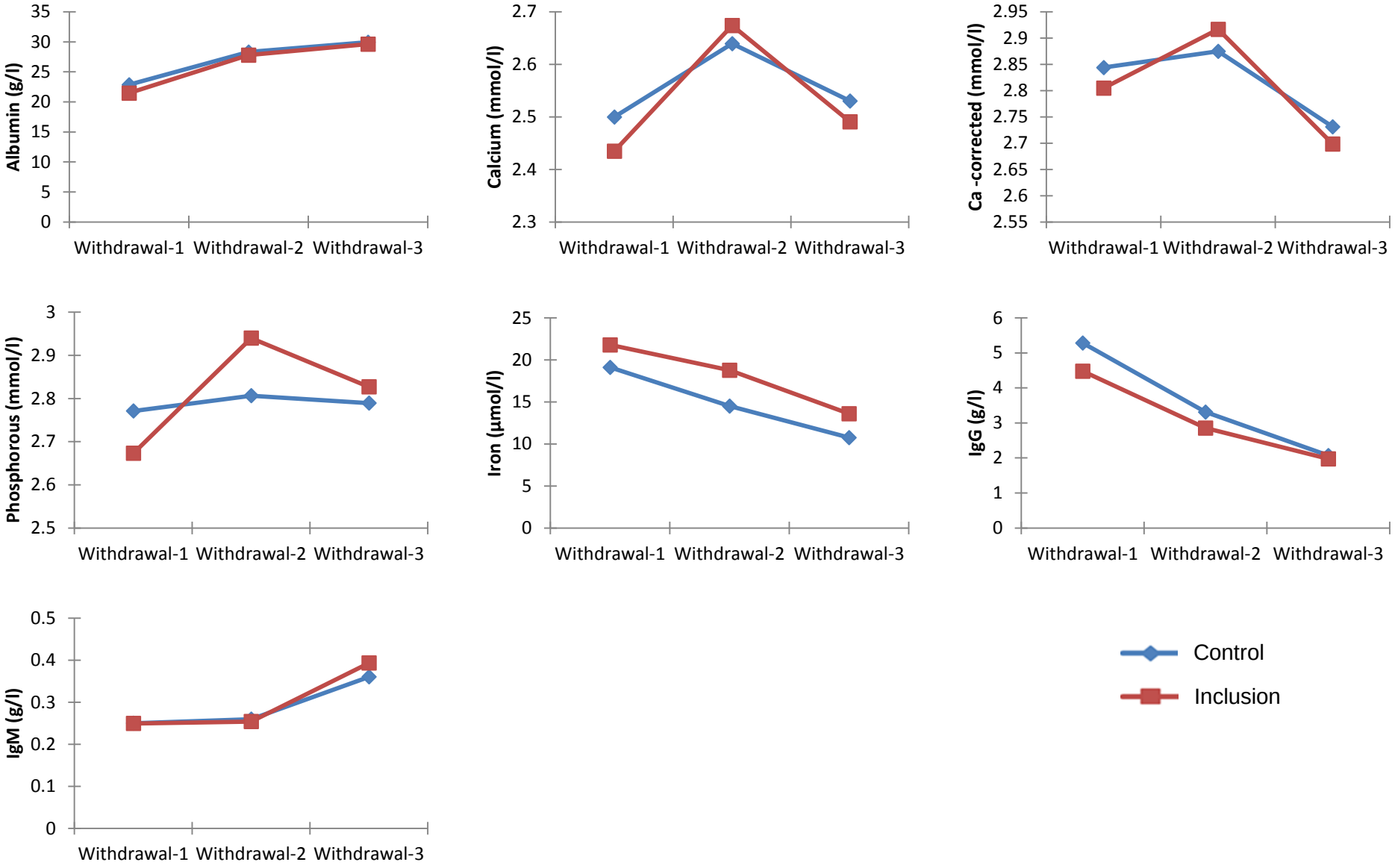


Figure 4:2 Average blood concentrations of the various biochemical parameters caused by withdrawal and treatment interaction.

4.5 Conclusion

There are many factors that have an effect on the blood parameters of the animal as described by various authors. These include sample collection and analysing techniques (Lippi *et al*., 1989; Banfi *et al*., 2007), stress (Minka and Ayo, 2007), diet (Etim *et al*., 2014), season (Chmielowiec-Korzeniowska *et al*., 2012) and physiological factors, such as genetics, age, sex and physiological stage (Tumbleson and Scholl, 1981). Factors within each of these categories are also noted, as the diet of the piglet includes both milk and creep feed. The effects of milk varying according to composition, absorption and utilization of the milk by the gut of the piglet (Inoue *et al*., 1980), as well as parity of the sow, genotype and season (Inoue *et al*., 1980; Bland and Rooke, 1998).

All these factors, along with the key limitations experienced during the data collection of the trial (dilution effect, sample collection mediated stress and antibiotic administration) and the very limited inclusion (9.66 g/piglet) of the BSFLM, made it incredibly difficult to make valid conclusions. As blood parameter values achieved in the current study may be correlated with the difference in ingredient inclusions and not the actual inclusion of the BSFLM itself, as its inclusion was limited to only 3.5% of the total diet. This inclusion percentage being the maximum allowable amount of the BSFLM, as to achieve a formulation that met the piglets' requirements. In the results achieved, there were no significant differences ($P>0.05$) between the treatment diets, except in the MCV and MCH values. These values showed significant differences ($P<0.05$) between the two treatment diets, as the BSFLM diet had higher values for both parameters over the entire trial. However, if either the treatment diets' starting or intercept points for blood sampling period 1 were the same or held constant, then there would be no significant difference between the treatments. The BSFLM diet also showed a higher increasing trend for both the HGB concentration and HCT percentage between sampling periods when compared to the control diet. Although there was not statistical significance ($P>0.05$) between the two treatment diets for these specific parameters, there may be biological correlation between the BSFLM and the respective higher values. However, evidence of this is not clear and would require further studies to be performed to associate these findings with the BSFLM.

The conclusion from the current study that could be made is that the inclusion of BSFLM may be utilized as an alternative protein source in piglet diets, where its inclusion had no beneficial or undesirable effects on the blood parameters of the animals. Piglets in the current study showed no visual signs of illness, as there was no abnormalities in activity or daily manure textures. However, it is recommended that further studies should be conducted to improve the research methodology used in the current trial before any acknowledgeable conclusions are made.

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Chapter 5

The effect of *Hermetia illucens* (black soldier fly) larvae on the microbiology of piglet manure.

5.1 Abstract

The effect of *Hermetia illucens* (black soldier fly) larvae meal supplementation on the manure microbiology and texture of piglets of 28 litters was investigated utilizing a block design consisting of two treatments (a control diet with a 0% inclusion of larvae meal and an inclusion diet with 3.5% larvae meal of total volume). *Hermetia illucens* larvae meal supplementation in a four week phase-over feeding scheme achieved results that showed no significant differences (P>0.05) between the black soldier fly larvae meal (BSFLM) diet when compared to the control diet in terms of manure microbiology and texture. However, an unintended administration of antibiotics did have a significant influence on the results and thus limiting conclusions. Therefore, further studies should be conducted to discover any possible effects on manure microbiology and texture caused by BSFLM inclusion in piglet diets and to evaluate the possible pathogen control associated in a farming system.

Keywords: BSF, larvae meal, pig, manure, microbiology, bacteria

5.2 Introduction

The problem of waste management is a growing concern, especially with the ever increasing large quantities of manure biomass being produced within the agricultural sector (Newton *et al*., 2005). The pathogens associated with these large quantities pose as a potential health risk to both animals and humans if not managed properly (Sobsey *et al*., 1989; Roberts and de Jager, 2004). Manure and other wastes, such as urine, sloughed feathers, fur, skin and even respiratory secretions, of various agricultural livestock often contain high concentrations (millions to billions per gram) of bacteria and other pathogens (Guselle and Olson, 2001). Table 5:1 provides some of the pathogens that may be potentially present in animal wastes. Agricultural animals such as cattle and pigs, especially in production facilities that harbour hundreds of animals per capita, produce incredibly large quantities of concentrated manure that must be effectively managed to minimize the associated environmental and public health risks (Guselle and Olson, 2001). There are numerous methods adopted for the management of manure biomass and pathogen content and black soldier fly (BSF) larvae may serve as a possible alternative solution for both these problems (Newton *et al*., 2005; Bondari and Sheppard, 1987).

The agricultural sector has various direct waste products which include manure, harvest residues and waste from processing plants (blood, whey, rejected food etc.). Also secondary waste products that are generated from the commercial industry, which include that from abattoirs, food retailers and the fermentation industry. This waste is usually turned into compost and used as fertilizers, however with the increasing concern for sustainable fuels, it is utilized for the production of biogas (Abraham *et al*., 2007). Manure can serve as a potential source of nutrients for black soldier flies (BSFs) and there have been reports by various authors on the efficiency at which this waste can be converted

to a valuable protein source (Calvert and Martin, 1969; Newton *et al*., 2005; St-Hilaire *et al*., 2007; Sealey *et al*., 2011; Pieterse, 2014; Pieterse *et al*., 2014). Newton *et al*. (2005) reported that BSF larvae reduced 55 kg of fresh manure dry matter to 24 kg of digested manure dry matter within 14 days, which is of a 56% reduction in the waste product. The presence of BSF larvae in manure also has many benefits, which include decreasing the moisture content (Calvert and Martin, 1969), odour (Teotia and Miller, 1974) and the volume and weight of the would-be waste organic matter (Sheppard *et al*., 2002). Other advantages include improving the texture of the manure (Teotia and Miller, 1974) and causing a significant reduction in *Escherichia coli* and *Salmonella enterica* by modifying the microbial flora of the manure (Bondari and Sheppard, 1987). El Boushy (1991) reported that there are various factors that have a significant influence on the chemical composition of manure, these include animal species, age, feeding ration and the amount of undigested feed present in the manure. Storage time of manure also has an influence on the chemical composition, where a reduction in the crude protein content is expected with an increase in the storage time (Flegal *et al*., 1972). Thus, it would be necessary to look into possible methods of nutrient binding/capturing to limit these nutrient losses with the increase in storage time.

Table 5:1 Some pathogens potentially present in animal wastes (Adapted from Sobsey *et al*., 1989).

Virus groups:	Hepatitis E virus (pigs), Reoviruses, Rotaviruses, Adenoviruses*, Caliciviruses*, Influenza viruses (Orthomyxoviruses)*
Bacterium groups:	*Salmonella* spp., *Campylobacter* spp., *Escherichia coli* **, *Aeromonas hydrophila***, *Yersinia enterocolitica, Vibrio* spp., *Leptospira* spp., *Listeria* spp., *Staphylococcus* spp.*
Parasites (Protozoans):	*Cryptosporidium parvum, Giardia lamblia* and *Balantidium coli*

*Humans and animals (including pigs) usually have distinct strains of these viruses, but not always.

**Some strains of these bacteria are non-pathogenic and others are pathogenic. The extent to which pathogenic strains occur in animal wastes varies with the animal species and other factors.

The nutrient recirculation organisms can potentially reduce the build-up of these waste products, specifically from the agricultural sector, as they are able to utilize these products as a feed source (Calvert and Martin, 1969; Newton *et al*., 2005; St-Hilaire *et al*., 2007; Sealey *et al*., 2011; Pieterse *et al*., 2014). The purpose of this study was to evaluate the effect of the inclusion of *H. illucens* larvae meal in the diets of the piglets on the manure pathogen content, as well as on manure texture.

5.3 Research design and methodology

5.3.1 Animals and diets

For this trial, the same 28 litters utilized in Chapter 3 and 4 were used for data collection, therefore the animals were managed as described in Chapter 3 (animal selection, housing, experimental diets, etc.).

The same piglet that was utilized for the collection of individual data in Chapter 3 and 4 was used for manure collection, thus, 28 piglets (14 per treatment) were utilized for this specific trial. A reminder that these piglets were those that fell closest to the average mass per piglet of their respective litters, and were used as a representation of their litter.

5.3.2 Experimental design and trial procedure

Three hundred and fifteen piglets were among the 28 litters utilized for the manure microbiology trial, where each litter was with its mother in the farrowing pen. The 28 litters were placed into two blocks according to the respective sows' farrowing dates, where the two treatments were administered with eight replications per treatment with an average of 11 piglets per replicate for block 1 and six replications per treatment with an average of 11 piglets per replicate for block 2. The average piglet mass for each litter was calculated and the piglet that was within the closest range to this average was selected for the collection of individual data as a representation for its respective litter. Thus, 28 piglets were used for sample collection with 14 replications per treatment.

5.3.3 Data collection and analyses

Manure swabs were taken from the anus, utilizing Sterilin sterile wooden applicator cotton tipped swabs, and performed at 9 (before treatment diets were administered) and 26 days of age (end of trial). The swabs were placed on ice immediately and stored at -20°C within 5 h after collection until the time of testing. Initially, the collection of manure matter samples was to be collected and tested, however, saw dust was administered to the pens on a daily basis as part of the normal routine on this farm to prevent or limit contact of the sow and piglets with the manure for hygiene purposes; limit possible pathogen transmission. Tests were performed at the Food Science Department at Stellenbosch University for the bacterial groups of *Escherichia coli (E. coli)*, *Staphylococcus (Staph.)*, *Salmonella* and *Listeria,* as well as a total viable count (TVC) which includes all traces of bacteria present in the manure samples. Table 5:3 provides a basic visual of the experimental procedures utilized for the quantification of bacterial groups.

Table 5:2 Experimental procedures used for the quantification of the bacterium groups.

Parameter	Method	Counts
Total viable count (TVC)	Aerobic Petri-film	Dilutions: -4, -5, -6
Escherichia coli	Violet red bile agar (VRBA)	Dilutions: -2, -3, -4, -5, -6
Staphylococcus	Mannitol salt agar (MSA)	Dilutions: -1, -2, -3, -4
Listeria	Polymerase Chain Reaction (PCR)	Absent/ Present
Salmonella	Polymerase Chain Reaction (PCR)	Absent/ Present

The bacterial counts were determined by adapted methods according to the International Organization for Standardization (ISO), where the individual manure swabs were diluted in 9 mL of buffered peptone water (BPW) and mixed vigorously for 1 min using a Vortex Mixer VM-300 to ensure all manure matter was diluted in the solution (homogenised). The respective serial dilutions for the tests of each bacterium group were then formed by pipetting 1 mL of the prior dilution in a consecutive sequence with mixing in between transmissions, again to ensure proper dilution. *Escherichia coli* and *Staphylococcus* dilutions were plated in petri-dishes containing specific bacterial growth medium and placed in an incubator room at 37°C for 24 h and the bacterial colonies manually counted. The TVC was performed utilizing aerobic petri-films (3M), where 1 mL of the respective dilutions were pipetted onto the film and placed in an incubator at 30°C for 24 h and the bacterial colonies were again manually counted. The remainder of the BPW dilutions after plating were utilized for the testing for *Salmonella* and *Listeria* using a Polymerase Chain Reaction (PCR), which provided a simple absent or present indication for traces of the latter. The PCR primer sequences for *Salmonella* were: ST11, 5'AGCCAACCATTGCTAAATTGGCGCA3'; ST14,

5'TTTGCGACTATCAGGTTACCGTGG3'; and ST15, 5'GGTAGAAATTCCAGCGGGTACTG3'. The PCR primer sequences for *Listeria* were: LmonoF 5'CATTAGTGGAAAGATGGAATG3' and LmonoR 5'GTATCCTCCAGAGTGATCGA3'. *Salmonella* and *Listeria* are able to be plated and colonies counted, however, according to the South African legislation VPN/15/2010-01 there should be no traces of the latter present in the samples. Thus, a simple absent or present identification was sufficient.

Daily manure scoring was also performed from time of feed administration (10 days of age) to the end of the trial (28 days of age), where manure textures were provided with either a score of 1- solid, 2- semisolid, 3- semi-liquid or 4- liquid. The observations were done at the same time each day, just before new saw dust was administered to the pens. Piglets were grouped according to treatment (14 litters per treatment) and cumulative frequency of each score was calculated as a percentage for each litter. Example: Litter 1 achieved a frequency of 8, 3, 3 and 2 of score 1, 2, 3 and 4, respectively, over the 16 days of feed administration. Percentage values were then calculated accordingly: 8/16, 3/16, 3/16 and 2/16 = 50%, 18.75%, 18.75% and 12.5%, respectively. Piglets were grouped according to treatment and overall percentage of scores was calculated for each treatment diet (addition of individual litters' percentages divided by number of litters, per treatment).

Note: The unintended administration of antibiotics interfered with the results, as can been seen in Table 5:4. These antibiotics included the oral dose of Baycox which acts specifically against the incidence of coccidiosis, as well as an injectable dose of Draxxin specifically for swine respiratory disease (SRD). Both of these antibiotics were administered at 3 days of age.

Assumptions

I. Normality between the piglets within a litter.

II. Normality between the litters.

5.3.3.1 Statistical analysis

Data were analysed using the PROC GLM of SAS for Windows Version 9.3 (statistical software). The statistics were done by using analysis of covariance with least square means (LSM) calculated with Bonferroni *post hoc* test. A probability of $P<0.05$ was used to determine significance. A full model was initially analysed where the main factor of parities was tested with farrowing dates included as blocks. Age (included as collection periods) and number of piglets per litter were also included as co-variables in the different models used for analysis. Due to parities, blocks and number of piglets per litter not having significant effect, these were excluded from the final model. Breed was also excluded due to very few Landrace animals included in the experiment. The final model included the variables of age and treatment. Repeated measures of ANOVA with Bonferroni *post hoc* test were completed using PROC MIXED. Manure bacterial concentrations were analysed by means of PROC GLM for treatments with each collection period being analysed separately. Graphs and charts have been included to provide a graphical representation of the data collected for both pathogen counts and the manure scoring.

5.4 Results and discussion

5.4.1 Manure microbiology

Table 5:4 summarizes the manure microbiology results achieved during the piglet performance trial. There were no significant differences (P>0.05) between the two treatment diets observed within collection periods, however, there were significant differences between collection periods which is correlated with the antibiotics that were administered to the piglets. A sample of the BSFLM was also tested as they are carriers of certain bacteria due to the substrate on which they are grown (Results also seen in Table 5:4). Bacterial counts were measured in colony forming units (CFU) per millilitre.

Note: The dilution selected for the representative counts in Table 5:4 for each bacterial group was selected according to the number of countable colonies, where if there were too numerous to count (TNTC) then the next consecutive dilution was considered until all colonies could be accurately quantified as this allowed for proper relative comparison.

Table 5:3 Manure microbiology results (CFU/mL).

Col.[a]	Diet	TVC	*Staph.*	*E.coli*	*L*[b]	*S*[b]
1	**Control**	72.50 ±52.97 x 10^4	44.17 ±25.20 x 10^1	10.17 ±12.61 x 10^3	A	A
	BSFLM	28.00 ±45.69 x 10^4	40.50 ±24.57 x 10^1	1.67 ± 2.25 x 10^3	A	A
2	**Control**	4.67 ± 6.02 x 10^4	4.17 ± 5.88 x 10^1	<10^3	A	A
	BSFLM	2.20 ± 4.38 x 10^4	13.60 ±12.42 x 10^1	<10^3	A	A
Black soldier fly larvae		<10^4	<10^1	<10^3	A	A

Col. = Collection period; BSFLM = Black soldier fly larvae meal; L = *Listeria*; S = *Salmonella*.
[a]Collection 1 performed at 10 days of age before feed administration and collection 2 at 28 days of age (end of trial).
[b]Tests run by PCR machine indicating 'Yes' or 'No' for traces of the bacterium tested (A = Absent; P = Present).

There was no significant differences between the control and BSFLM diets for the counts for TVC, *Staphylococcus* and *E. coli* at each collection period (Table 5:4). *Listeria* and *Salmonella* were absent at both collection periods for both treatment diets. It can be seen in Table 5:4 that there was a count value of <10^3 CFU/mL for counts of *E. coli* in both the control and inclusion diets at the second collection. This implies that there was a count of less than 1000 colonies present in the samples and not the absence of *E. coli* in the respective manure samples. This is explained by the fact that antibiotics were administered to the piglets, where both the oral dose of Baycox and injectable dose of Draxxin were administered at 3 days of age. Baycox was administered to prevent specifically against coccidiosis and Draxxin specifically for the prevention of swine respiratory disease (SRD), however, this may have had an influence on other bacterium present in the piglet and manure. Nightingale (1997) reported that Draxxin (tulathromycin) has antimicrobial effects that have not yet been characterized, but may be bactericidal against some pathogens. It may also exhibit a post-antibiotic effect, but to what extent and to what duration is dependent on both the drug administration and pathogens present (Nightingale, 1997). Draxxin is a long lasting antibiotic and animals may take approximately 3 to 5 days to respond after its administration (as reported by

Drugs.com, available at http://www.drugs.com/vet/draxxin-injectable-solution-can.html). Therefore, although it was administered at 3 days of age in the current study it may have only started taking full effect at approximately 8 days of age. This may explain why bacterium traces were very low at the second collection when compared to the first collection. Furthermore, there may have been bacterial retention by the swabs which may have hindered the subsequent number of colonies cultured on the respective agar plates, where Collee *et al*. (1973) reported that the apparent loss may not be primarily attributed to the inactivation on the swab but rather a retention of the organisms on the swab. Kabayiza *et al*. (2013) reported contradictory to the latter, that both manure matter samples and rectal swabs showed no significant differences when counts were compared. This could be attributed to the fact that Kabayiza *et al*. (2013) utilized real-time PCR-based identification and quantification and Collee *et al*. (1973) plated quantification. Subsamples of the BSFLM were also tested for bacterial counts, as these insects are likely carriers of certain bacterium groups and may transfer these bacterium to a host through consumption. Where, the bacterial counts within the fly larvae may vary depending on the substrate on which they are grown. In the current study the larvae were grown on kitchen waste which may explain why there were no traces of the respective bacterium in the dilutions tested, however, there may possibly be counts in larvae grown on manure as bacterium are present in manure matter; but research into the latter has not yet been conducted. There were also no traces of *Listeria* and *Salmonella* in the first and second collection period of the manure samples or in the BSF larvae meal. Figure 5:1 provides a graphical representation of the results achieved in the current study.

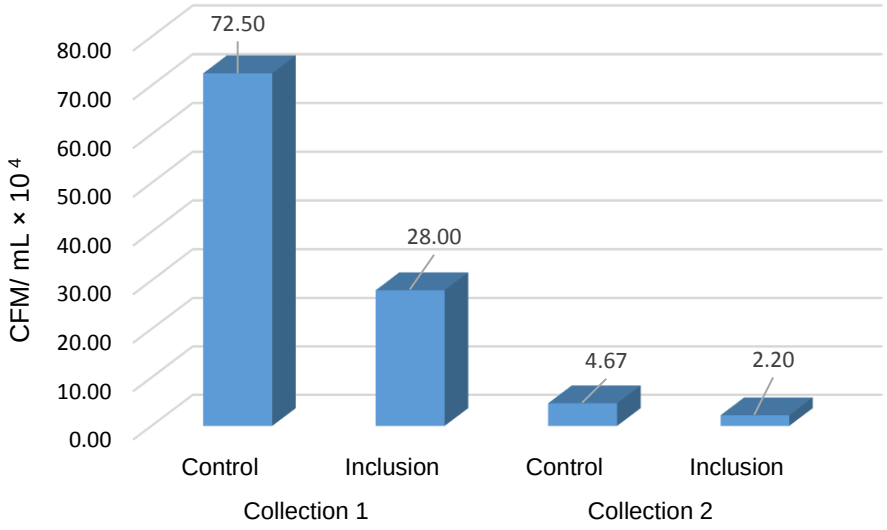

Figure 5:1 Least square means of the results achieved for total viable count (TVC).

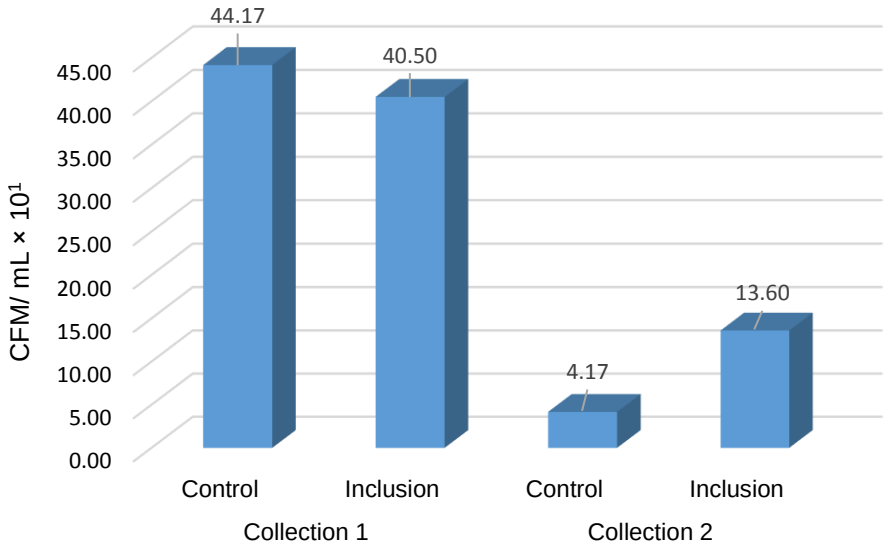

Figure 5:2 Least square means of the results achieved for *Staphylococcus* (CFU/mL).

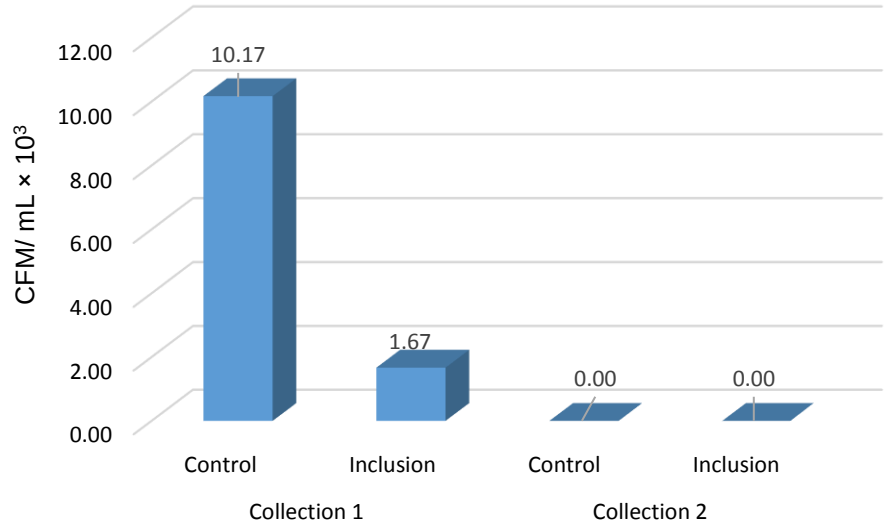

Figure 5:3 Least square means of the results achieved for *E.coli* (CFU/mL).

5.4.2 Manure scoring

Results achieved for daily manure scoring in the current study showed that there was no significant differences between the two treatment diets from time of feed administration (10 days of age) to the end of the trial (28 days of age). The pie charts in Figure 5:2 (Control diet) and Figure 5:3 (BSFLM inclusion diet) provide a visual for the manure scoring for those litters that received the treatment diets.

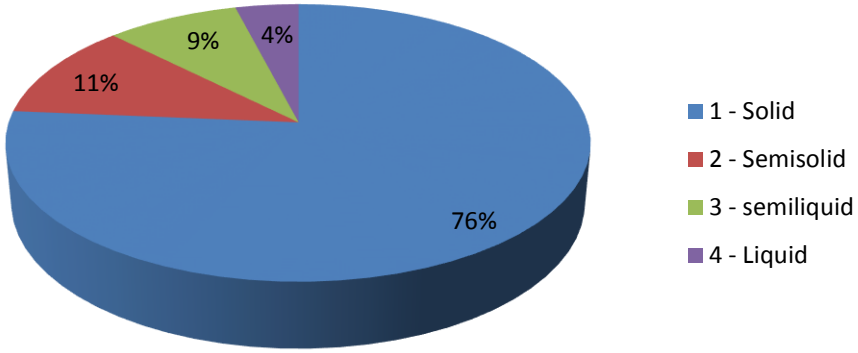

Figure 5:4 Cumulative manure scoring results of those litters that received the control diet from feed administration (10 days of age) to the end of the trial (28 days of age).

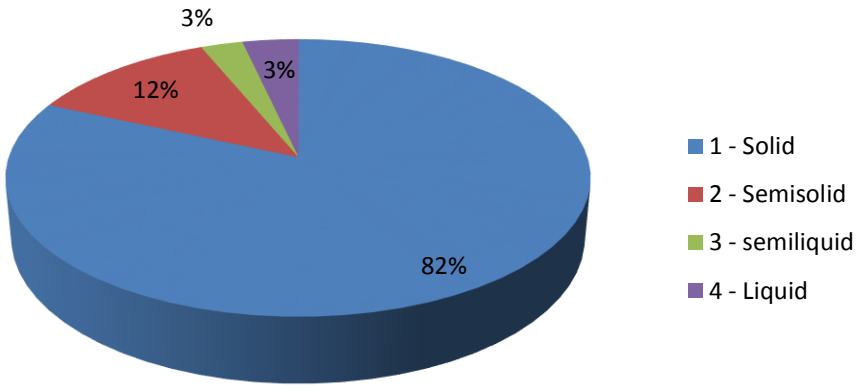

Figure 5:5 Cumulative manure scoring results of those litters that received the inclusion diet from feed administration (10 days of age) to the end of the trial (28 days of age).

5.5 Conclusion

Limited conclusions could be made from this investigation due to an unintended administration of antibiotics to the piglets during the current trial. Thus, as to what effect the BSFLM had on the manure microbiology (bacterial shedding load) and manure texture could not be evaluated. Further studies would be required to discover the effects the larvae meal may have on such manure parameters and consequent pathogen control within a farming system.

5.6 References

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Chapter 6

General conclusion

Literature has provided that black soldier fly larvae meal (BSFLM) has been tested and proved to have significant influence in production and/or health aspects of other production animals, such as poultry, fish and adult pigs (Chapter 2). It has also been considered as a potential solution to other economical and environmental hazards, such as waste management. *Hermetia illucens* (black soldier fly) larvae meal has proved in this study to be a good-quality, sustainable protein source that can be efficiently utilized as an alternative to traditional sources currently used in the diets of piglets at an inclusion rate of 3.5%. The proximate analysis of the BSFLM showed that it contained 35.9% crude protein, 48.1% ether extract, 6.5% crude fibre and 7.8% ash (minerals) (Chapter 3). The larvae for the current study were dried, but not defatted, thus the fat content was relatively high. The reason for this was that the larvae were harvested at a time in which they were still active feeders and had not yet reached the stage of metamorphosis in which the fat reserves are utilized as an energy source. The formulation of the inclusion feed limited the BSFLM content to a maximum of 3.5% of the total volume, as this met the piglets' requirements. This limitation was caused by the high fat content of the larvae meal as the gastrointestinal tract of the piglet during the early stages of life has not yet fully developed to efficiently digest fat.

Data reported that BSFLM inclusion did not have a significant influence ($P>0.05$) on the litter average live weights, feed intakes and ADGs when compared to a control diet (0% BSF larvae inclusion). Thus, the results of the production study indicated that BSFLM inclusion in piglet diets sustained and supported normal piglet growth and development (Chapter 3). It is reported that in the current study that larvae meal also had no significant effects ($P>0.05$) on the blood parameters of the piglets. Therefore, results indicate that the inclusion of BSFLM in piglet diets had no noteworthy immunological influence or health effect (positive or negative) and can thus be utilized for piglet production, while maintaining normal blood parameters. However, the inclusion diet did show superior increasing values for both haemaglobin (HGB) and haematocrit (HCT) over the trial (Chapter 4) and, although not significantly different, there may be biological value as HCT is the volume percentage of RBCs and HGB is the iron-containing oxygen-transport metalloprotein within RBCs. Thus, these higher values may be correlated with better oxygen binding capacity and transport of the oxygen to the tissues of the body. Although, this phenomena could also be an indication of immunological stress. However, due to the issues of the dilution effect, sample collection mediated stress and administration of antibiotics experienced during the data collection of this trial, further research is needed to validate this part of the study.

In regards to the effect of BSFLM on the manure microbiology (bacterial shedding load) and texture (Chapter 5), acknowledgeable conclusions could not be discussed as an unintended administration of antibiotics interfered with the results. However, results achieved in the current study showed no significant difference ($P>0.05$) between the treatment diets. Further research is recommended to evaluate the possible effect of BSFLM on such manure parameters. Nevertheless, the use of insect meals in animal production systems will lead to a decrease in organic waste matter through vermicomposting and, in the process, yield larvae, pupae or pre-pupae that will increase protein availability for its use in animal nutrition. This will contribute to food security as it will reduce the

utilization of specific crop products in animal feeds, so that they may be used in other markets (specifically for human consumption).

It can therefore be concluded that BSFLM is a viable protein source for its use in pig production, without compromising production efficiency. Depending on the economies of scale and the availability of the larvae meal within the animal feed market, dried and full-fat larvae can be included as a protein source at a level of 3.5% of the total volume of a piglet diet. The real challenge that may prove difficult to overcome is to mass produce BSF larvae in sufficient quantities of similar quality for its use as an animal feed in production systems.

Further research recommendations

I. Ensure that treatment feeds are palatable, which provides the piglets with enough desire or incentive to consume sufficient feed for optimal growth and development. As well as to consume enough of the treatment diets for a greater chance of possible responses.
II. It is recommended to investigate the inclusion of defatted BSFLM, as piglets have not yet developed the ability to fully digest the fat content of the diet. Furthermore, the use of defatted larvae meal would enable a larger inclusion of the larvae meal. This in turn could allow for the investigation of various inclusion levels to discover their respective influences on the performance of the piglet, as well as discover an optimal inclusion level within the piglets' diet.
III. In the current study only the use of larvae meal was investigated in the diets of piglets, but much more research is recommended on the use of the possible inclusion of pupae meal in piglet diets.
IV. Experienced vet to ensure correct blood sample collection and handling techniques. Also, the adaption of the trial animals to handling and blood collection methods before the onset of blood collection as to limit stress which may otherwise interfere with results (handle 2 or 3 days a week for one to two weeks prior collection).
V. Controlled research enviroment: smaller groups, more repetitions, no administration of antibiotics, positive and negative control.
VI. Trials to be performed to compare the potential of BSF larvae grown on different feed substrates, as a vector for the transmission of different bacterium from being administered as a feed source to a host animal through consumption.
VII. Research is also required on the use of larvae and pupae meal in the diets of weaner, grower and finisher pigs to determine the effect of these meals on pigs at different production stages. This would also allow for larger intakes and the FCR to be calculated, as there would no longer be the influence of suckling milk from the sow.
VIII. Research into the use of insect meals in other species including other monogastric animals, ruminants and aquaculture is also warranted. This research should also focus on the bio-availability of minerals, palatability, susceptibility to heat damage and rumen degradability.